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


MedImmune Vaccines Inc., 297 North Bernardo Avenue, Mountain View, CA 94043, USA

Restricted replication in the respiratory tract of rhesus monkeys is an intrinsic property of bovine parainfluenza virus type 3 (bPIV-3) strains. This host range phenotype of bPIV-3 has been utilized as a marker to evaluate the attenuation of bPIV-3 vaccines for human use. Two safety, immunogenicity and efficacy studies in primates evaluated and compared three human parainfluenza virus type 3 (hPIV-3) vaccine candidates: biologically derived bPIV-3, a plasmid-derived bPIV-3 (r-bPIV-3) and a chimeric bovine/human PIV-3 (b/hPIV-3). These studies also examined the feasibility of substituting Vero cells, cultured in the presence or absence of foetal bovine serum, for foetal rhesus lung-2 (FRhL-2) cells as the tissue culture substrate for the production of bPIV-3 vaccine. The results demonstrated that (i) Vero cell-produced bPIV-3 was as attenuated, immunogenic and efficacious as bPIV-3 vaccine grown in FRhL-2 cells, (ii) plasmid-derived bPIV-3 was as attenuated, immunogenic and efficacious as the biologically derived bPIV-3 and (iii) the b/hPIV-3 chimera displayed an intermediate attenuation phenotype and protected animals completely from hPIV-3 challenge. These results support the use of bPIV-3 vaccines propagated in Vero cells in human clinical trials and the use of b/hPIV-3 as a virus vaccine vector to express foreign viral antigens.

INTRODUCTION

Human parainfluenza virus type 3 (hPIV-3), a causative agent of lower respiratory tract diseases, such as virus pneumonia and bronchiolitis in infants and children, is closely related to bovine parainfluenza virus type 3 (bPIV-3) (Welliver et al., 1986). Both are members of the family Paramyxoviridae. There is currently no licensed vaccine for hPIV-3. The amino acid sequences of the haemagglutinin–neuraminidase (HN) and fusion (F) proteins of bPIV-3 and hPIV-3 are greater than 75% related (Bailley et al., 2000), the bovine and human viruses are approximately 25% antigenically related by cross-neutralization (van Wyke Coelingh et al., 1988) and at least five neutralization epitopes on the HN and F proteins are shared between the two viruses (Coelingh et al., 1986; Klippmark et al., 1990). In addition, the major viral non-glycoproteins N, M and L are greater than 85% related between bPIV-3 and hPIV-3. Neutralizing antibodies to HN and F play a central role in resistance to PIV-3 infection and illness in humans, and T-cell response to these may be important for resolution of infection. The high degree of relatedness between the two viruses provided the rationale of using bPIV-3 as a live virus vaccine to stimulate both humoral and cellular immunity capable of protecting humans from hPIV-3 disease. Another live attenuated hPIV-3 vaccine candidate being considered for human clinical studies was generated by reverse genetics, yielding a recombinant bPIV-3 (r-bPIV-3). r-bPIV-3 is phenotypically identical to the biologically derived bPIV-3 (Haller et al., 2000, 2001). The bPIV-3 vaccine strain (bPIV-3/Kansas/15626/84) used here was isolated in 1984 from a calf with pneumonia in Kansas and was shown to be immunogenic in hamsters (Haller et al., 2000) and in Old World and New World non-human primates (van Wyke Coelingh et al., 1988). Compared to bPIV-3, bPIV-3 was approximately 100- to 1000-fold restricted for replication in the lower (LRT) and
upper respiratory tract (URT) of rhesus monkeys. This host range restriction was correlated with reduced virulence in humans and a live bPIV-3 vaccine was shown to be safe, attenuated and immunogenic in children (Karron et al., 1995, 1996). These results provided the basis for development of bPIV-3 as a vector to express foreign viral antigens. Initially, the bPIV-3 F and HN genes were substituted with those of hPIV-3 to generate a chimeric bovine/human PIV-3 (b/hPIV-3) vaccine candidate, with the goal of inducing an immune response that is antigenically homologous with respect to the hPIV-3 HN and F proteins, which are targets for neutralizing antibodies (Haller et al., 2000, 2001). Since the attenuation determinants reside in multiple bPIV-3 genes and b/hPIV-3 contains 78% of the bPIV-3 genome, b/hPIV-3 is expected to retain the attenuation phenotype (Schmidt et al., 2000).

A live attenuated bPIV-3 vaccine was evaluated in a phase 2 clinical trial (Lee et al., 2001; Greenberg et al., 1999) in which the vaccine was administered intranasally, concurrently with other routine vaccinations, to infants at 2, 4 and 6 months of age, followed by a booster immunization at 12–15 months of age. This trial demonstrated that bPIV-3 was safe and well tolerated in infants and that the immunization elicited a humoral antibody response to bPIV-3 that cross-reacted with hPIV-3. There were no significant differences between vaccine and placebo groups with respect to rates of fever >101°F or other post-vaccination adverse events (Greenberg et al., 1999). Since the bPIV-3 vaccine used in this phase 2 trial was prepared on foetal rhesus lung-2 (FRhL-2) cells, it was necessary to identify cell substrates suitable for commercial production of bPIV-3, as routine vaccine manufacture using FRhL-2 cells was not feasible. After testing bPIV-3 replication in 10 cell lines, Vero cells were identified as the cell substrate for vaccine production based on vaccine yield considerations. Although no live virus vaccine manufactured in Vero cells has yet been approved by the Food and Drug Administration (FDA) for use in humans, the FDA has issued recommendations for producing vaccines in Vero cells.

Two primate studies were performed to characterize attenuation, immunogenicity and efficacy of potential PIV-3 vaccines following propagation in either FRhL-2 or Vero cells. Study A compared bPIV-3 vaccine manufactured in Vero cells [bPIV-3(V)] to that produced in FRhL-2 cells [bPIV-3(F)] with respect to antigenicity in serological assays as well as immunogenicity and attenuation in non-human primates. In study B, the feasibility of using Vero cells grown in medium without foetal bovine serum (FBS) for PIV-3 vaccine manufacture was evaluated to reduce the possibility of contracting bovine spongiform encephalopathy. Also in study B, attenuation, immunogenicity and efficacy of plasmid-derived r-bPIV-3 and b/hPIV-3 were studied to evaluate their potential application as vaccine vectors.

The results of the two primate studies showed that the attenuation, immunogenicity and efficacy profiles of bPIV-3(V) and bPIV-3(F) vaccines were similar and supported the use of Vero cells for vaccine manufacture. The growth of bPIV-3, r-bPIV-3 or b/hPIV-3 in serum-free (SF) Vero cells did not affect vaccine yield, virus replication, immunogenicity or efficacy in primates. The data presented here also showed that r-bPIV-3 and b/hPIV-3 were attenuated and efficacious in primates. They represent novel virus vectors and vaccine candidates to be evaluated in future clinical studies.

METHODS

Cells and viruses. Vero cells obtained from the WHO cell bank were maintained in DMEM supplemented with 2 mM L-glutamine, non-essential amino acids (NEAA) and 5% FBS. FRhL-2, Madin–Darby bovine kidney (MDBK) and rhesus monkey kidney (LLC-MK2) cells were cultured in DMEM containing 2 mM L-glutamine, NEAA, antibiotics and 10% FBS. All media components were purchased from HyClone Laboratories. SF Vero cells were grown in OptiPRO SFM medium (Invitrogen) supplemented with 4 mM L-glutamine and antibiotics.

bPIV-3 (Kansas/15626/84), hPIV-3 (JS) and hPIV-3 (Washington/47885/57) were propagated in Vero and FRhL-2 cells. Cells were infected with bPIV-3 or hPIV-3 at an m.o.i. of 0·001 TCID50 per cell and incubated at 37°C. Culture medium was centrifuged at 600 g for 10 min. The supernatant was stabilized by adding 10 x SPG (2·18 M Sucrose, 0·038 M KH2PO4, 0·072 M K2HPO4 and 0·054 M L-glutamate) to a final concentration of 1 x bPIV-3, hPIV-3, r-bPIV-3 and b/hPIV-3 were grown in SF Vero cells. Briefly, Vero cells were infected at an m.o.i. of 0·01 TCID50 per cell and overlaid with Opti-MEM I. At 3 days post-infection, cells and supernatants were collected, SPG was added and virus stocks were stored at −70°C. Virus titres were determined by TCID50 assays.

Rhesus monkey studies

Study A. Rhesus monkeys (1–4 years old; 3–5 kg) were pre-screened for PIV-3 antibodies by haemagglutination inhibition assay (HAI) and seronegative animals were randomly assigned to five groups (four monkeys per group). Groups of monkeys were anaesthetized with a ketamine/valium mixture and vaccinated intratracheally and intranasally via drops with bPIV-3(F) vaccine, bPIV-3(V) vaccine, hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2 x 105 TCID50 bPIV-3(F), bPIV-3(V), hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2 x 105 TCID50 of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose.
Opti-MEM. On day 28, all animals were challenged intratracheally and intranasally with $2 \times 10^5$ TCID$_{50}$ bPIV-3(F) (Vero cells). NP swabs were collected daily for 11 days and TL specimens were collected on days 1, 3, 5, 7 and 9 post-immunization and post-challenge. Blood samples were collected on days 0, 7, 14, 21, 28, 35, 42, 49 and 56.

Quantification of virus shedding. bPIV-3 and hPIV-3 present in the primate NP and TL specimens were quantified by TCID$_{50}$ assays using MDBK, LLC-MK2 or Vero cells. Previous studies had indicated that MDBK cells were sensitive for bPIV-3 replication and LLC-MK2 cells were sensitive for hPIV-3 replication. To confirm that the CPE observed was produced by virus infection and not mucus and cell debris present in the NP and TL samples, the TCID$_{50}$ assay was modified to include haemadsorption with guinea pig (gp) erythrocytes. Haemadsorption was recorded visually and virus titres were calculated according to the Karber modification of the Reed–Muench equation (Reed & Muench, 1938). Mean peak virus titres represent the mean of the peak TCID$_{50}$ titre measured on any of the 12 days following dose 1, dose 2 or challenge.

HAI assay. HAI assays were performed by incubating serial 2-fold dilutions of monkey serum at 25°C for 30 min with 8 HA units per 0.05 ml of either bPIV-3 or hPIV-3. Subsequently, gp red blood cells were added to each well, incubation was continued for 90 min and each well was then observed for haemagglutination. HAI titres were expressed as the reciprocal of the highest dilution of antiserum that inhibited virus-mediated agglutination of erythrocytes. To facilitate calculation of the geometric mean titre (GMT), HAI titres of $\leq 1:4$ (the lowest serum dilution tested) were assigned a titre of 1:2.

Microneutralization assays. Microneutralization assays were performed on MDBK, LLC-MK2 or Vero cells. Serial 2-fold dilutions of serum, starting at 1:4, were incubated at 37°C for 60 min with 100 TCID$_{50}$ of either bPIV-3 or hPIV-3. Then, 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 CPE. Neutralization titres were expressed as the reciprocal of the highest serum dilution that inhibited CPE. Neutralization antibody titres of $\leq 1:4$ (the lowest serum dilution tested) were assigned a titre of 1:2.

PIV-3 IgA ELISA. Glycoprotein extracts of bPIV-3/Kansas/15626/84 and hPIV-3/Washington/47885/97 were prepared as follows. bPIV-3- or hPIV-3-infected MDBK cell supernatants were centrifuged at 100 000 g to pellet the virus, which was then resuspended in PBS, overlaid on a 30% sucrose cushion and centrifuged at 100 000 g for 90 min. The pellet was resuspended in PBS, treated with an equal volume of 1% Nonidet P-40 (in PBS) for 4 h at room temperature and centrifuged at 100 000 g for 90 min. The extracted HN glycoprotein supernatant was stored frozen. Serial 4-fold dilutions of serum were added to 96-well plates that had been coated previously with glycoprotein extracts of bPIV-3 or hPIV-3. Bound monkey IgA was detected by the addition of horseradish peroxidase-conjugated sheep anti-human IgA antiserum, followed by 3,3’5,5’-tetramethylbenzidine substrate. The absorbance of the substrate was read spectrophotometrically at 450 nm. The absorbance values obtained with rhesus monkey serum samples were compared to a standard curve of a human reference IgA antiserum. The amount of bound serum IgA was expressed as units of IgA ml$^{-1}$. Test serum samples that produced a signal in the IgA ELISA that was $\leq 7-8$ units IgA ml$^{-1}$ (the lowest value represented in the standard curve of the reference antiserum) was assigned a titre of 7-8 units IgA ml$^{-1}$.

RESULTS

Attenuation and immunogenicity in rhesus monkeys after a single dose of bPIV-3 manufactured in Vero or FRhL-2 cells (Study A)

Vaccine safety and efficacy were evaluated in a rhesus monkey replication model of attenuation. To determine whether the attenuation phenotype associated with bPIV-3 would be preserved in vaccines produced in Vero as well as FRhL-2 cells, virus shedding after the first immunization (dose 1) was measured and replication of bPIV-3(F) vaccine was compared to that of hPIV-3(F), and replication of bPIV-3(V) vaccine was compared to hPIV-3(V).

Following primary vaccination with bPIV-3(F), monkeys shed for 9 days in the nasopharynx, displaying a mean peak titre of $10^{3.1}$ TCID$_{50}$ ml$^{-1}$, and for 8 days in the trachea, with mean peak titres of $10^{5.6}$ TCID$_{50}$ ml$^{-1}$. In contrast, vaccination with non-attenuated bPIV-3(F) resulted in virus shedding for 10 days in the nasopharynx, showing mean peak titres of $10^{4.5}$ TCID$_{50}$ ml$^{-1}$, and for 9 days in the trachea, with peak titres of $10^{2.9}$ TCID$_{50}$ ml$^{-1}$ (Table 1). Thus, shedding of bPIV-3(F) was 25-fold lower in the nasopharynx and 16-fold lower in the trachea compared to hPIV-3(F). Monkeys that received bPIV-3(V) shed $10^{3.3}$ TCID$_{50}$ ml$^{-1}$ for 9 days in the nasopharynx and $10^{1.4}$ TCID$_{50}$ ml$^{-1}$ for 8 days in the trachea. In contrast, monkeys vaccinated with hPIV-3(V) shed $10^{0.1}$ TCID$_{50}$ ml$^{-1}$ for 9 days in the nasopharynx and $10^{5.2}$ TCID$_{50}$ ml$^{-1}$ for 7 days in the trachea (Table 1). The shedding of bPIV-3(V) was 63-fold lower in the nasopharynx and 79-fold lower in the trachea compared to hPIV-3(V).

The antibody responses induced in the animals upon immunization were studied by HAI, neutralization and IgA ELISA assays to determine whether the cell line used for manufacture had an influence on immunogenicity. All monkeys mounted a serum HAI antibody response to bPIV-3 (a ‘homologous’ antibody response) following the initial vaccination with either bPIV-3(F) or bPIV-3(V) (Table 2). Vaccination with bPIV-3(F) and bPIV-3(V) resulted in a homologous HAI GMT of 90.5 and 64.0, respectively. All vaccinated monkeys also mounted a HAI antibody response to hPIV-3 (a ‘heterologous’ antibody response). Vaccination with bPIV-3(F) and bPIV-3(V) resulted in a heterologous HAI GMT of 8.0 and 16.0, respectively (Table 2). Titres within 4-fold of each other in this serological assay are considered comparable.

The primary serum neutralizing antibody response was similar for both bPIV-3 vaccines tested (Table 3). bPIV-3(F) and bPIV-3(V) induced a homologous neutralizing antibody GMT of 51.6 and 70.8, respectively, in a neutralization assay that utilized bPIV-3 grown on FRhL-2 cells. The homologous GMT measured was also comparable for the two vaccines when the assay was performed using bPIV-3 prepared in Vero cells. Consistent with the serum HAI results, bPIV-3(F) and bPIV-3(V) induced a heterologous
neutralizing antibody GMT of 34.9 and 26.9, respectively, when the assay was performed with hPIV-3 grown on LLC-MK2 cells.

When the antibody response was measured using an IgA ELISA, the titres were similar (within 2-fold) for bPIV-3 vaccine produced in either FRhL-2 or Vero cells (Table 3). bPIV-3(F) and bPIV-3(V) induced a homologous IgA antibody GMT of 52.2 and 53.6, respectively, and a heterologous GMT of 10.0 and 20.1, respectively.

**Immune response of rhesus monkeys to a bPIV-3 vaccine booster dose (Study A)**

On day 28, all animals received booster vaccinations (dose 2), identical to the vaccines or viruses used for dose 1. bPIV-3(F) or bPIV-3(V) vaccines were administered to study whether the antibody response could be enhanced. bPIV-3 shedding was not detected except in one of four animals, which displayed low levels (10^1 TCID_50 ml^-1) of bPIV-3(V) shedding in the trachea. In contrast to the results with the bPIV-3 vaccine, hPIV-3(F) or hPIV-3(V) were not observed in either nasopharyngeal or tracheal specimens after dose 2 (data not shown).

The serum HAI GMT to bPIV-3 continued to increase after the booster dose of bPIV-3 vaccine produced in FRhL-2 or Vero cells. After re-vaccination with a second dose of bPIV-3(F) or bPIV-3(V), the homologous HAI GMT increased 1.4-fold (from 90.5 to 128.0 and from 64.0 to 90.5, respectively) (Table 2). Re-vaccination also resulted in a similar increase in the heterologous HAI GMT for both bPIV-3 vaccines tested (from 8.0 to 32.0 and from 16.0 to 22.6, respectively).

**Table 2. Serum HAI antibody response upon immunization of rhesus monkeys with bPIV-3 (F or V) and hPIV-3 (F or V) (study A)**

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>No. of animals</th>
<th>Serum HAI GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 (pre-dose)</td>
<td>Day 28 (post-dose 1)</td>
</tr>
<tr>
<td></td>
<td>hPIV-3</td>
<td>bPIV-3</td>
</tr>
<tr>
<td>bPIV-3(F)</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>bPIV-3(V)</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>hPIV-3(F)</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>hPIV-3(V)</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>Placebo</td>
<td>4</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Table 3. Serum neutralizing antibody and serum IgA ELISA responses upon immunization of rhesus monkeys with bPIV-3 (F or V) and hPIV-3 (F or V) (Study A)

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>No. of animals</th>
<th>GMT serum neutralizing antibody on day 28</th>
<th>GMT serum IgA antibody on day 28 (IgA units ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>bPIV-3 (FRhL)*,†</td>
<td>bPIV-3 (Vero)*,†</td>
</tr>
<tr>
<td>bPIV-3(F)</td>
<td>4</td>
<td>51·6</td>
<td>173·5</td>
</tr>
<tr>
<td>bPIV-3(V)</td>
<td>4</td>
<td>70·8</td>
<td>221·7</td>
</tr>
<tr>
<td>hPIV-3(F)</td>
<td>4</td>
<td>51·6</td>
<td>119·1</td>
</tr>
<tr>
<td>hPIV-3(V)</td>
<td>4</td>
<td>39·2</td>
<td>135·8</td>
</tr>
<tr>
<td>Placebo</td>
<td>4</td>
<td>2·0</td>
<td>2·0</td>
</tr>
</tbody>
</table>

*Antigen used in the neutralization or ELISA assay. Day 0, pre-dose; day 28, post-dose 1.
†Serum neutralizing antibody titres on day 0 were 2·0.
‡bPIV-3 and hPIV-3 used to isolate the glycoproteins were propagated on MDBK cells.
§The lowest value on the IgA ELISA standard curve of the reference antiserum is 7·8 IgA units ml⁻¹. Serum IgA ELISA titres on day 0 were 7·8 IgA units ml⁻¹.

Efficacy of bPIV-3 vaccine in rhesus monkeys against hPIV-3 challenge (Study A)

It was important to demonstrate that bPIV-3 vaccines produced in either FRhL-2 or Vero cells were efficacious in rhesus monkeys and protected from hPIV-3 challenge. Indeed, the only samples showing challenge virus shedding were collected from the placebo group. Placebo recipients shed challenge virus for 10 days in the nasopharynx with mean peak titres of $10^{4.1}$ TCID₅₀ ml⁻¹ and for 9 days in the trachea, where mean peak titres of $10^{3.6}$ TCID₅₀ ml⁻¹ were observed. In contrast, virus was not shed post-challenge in the nasopharynx of monkeys previously vaccinated with two doses of bPIV-3(F) or bPIV-3(V) (Table 1). Similarly, hPIV-3 challenge virus was not detected in tracheal specimens from these vaccinated animals, with the exception of a single sample that contained the maximum detectable level of virus ($10^4$ TCID₅₀ ml⁻¹). Challenge hPIV-3 was also not observed in either nasopharyngeal or tracheal specimens obtained from animals that had received two inoculations of bPIV-3(V) or hPIV-3(F).

Following hPIV-3 challenge, the serum HAI GMT was boosted in animals that had been previously vaccinated with bPIV-3 vaccine produced in either FRhL-2 or Vero cells (Table 2). The GMT to bPIV-3 doubled in animals previously vaccinated with bPIV-3(F) or bPIV-3(V), and the GMT to hPIV-3 increased 10-fold in animals previously vaccinated with either bPIV-3(F) or bPIV-3(V).

Attenuation and immunogenicity of r-bPIV-3 and b/hPIV-3 in rhesus monkeys (Study B)

Development of bPIV-3 as a vaccine vector necessitated the construction of an infectious cDNA of the bPIV-3 genome such that foreign antigens could be introduced at the cDNA level (Haller et al., 2000). We demonstrated that r-bPIV-3 was phenotypically identical to the biological bPIV-3 in vitro and in vivo using hamsters (Haller et al., 2001). However, hamsters do not represent a sensitive model for studying bPIV-3 attenuation. To show that r-bPIV-3 and b/hPIV-3, containing the hPIV-3 F and HN genes, retained the attenuation determinants, a rhesus monkey study was performed. Restricted replication of bPIV-3 in the LRT and URT of rhesus monkeys when compared to hPIV-3 can be correlated and used as an attenuation marker of bPIV-3 in humans. All of the virus stocks used in this study for immunization of the animals were grown in SF Vero cells.

In this study, r-bPIV-3 replicated to mean peak titres of $10^{3·6}$ and $10^{3·8}$ TCID₅₀ ml⁻¹ in the nasopharynx and trachea of immunized monkeys, respectively. These titres were comparable to those obtained with biological bPIV-3. Monkeys that received hPIV-3/JS shed $10^{5·0}$ and $10^{6·7}$ TCID₅₀ ml⁻¹ in the URT and LRT. Thus, shedding of r-bPIV-3 was 25-fold lower in the nasopharynx and 79-fold lower in the trachea compared to hPIV-3. b/hPIV-3 demonstrated an intermediate attenuation phenotype compared to r-bPIV-3 and hPIV-3. b/hPIV-3 replication was reduced 13-fold in the nasopharynx and 6-fold in the trachea of rhesus monkeys compared to hPIV-3 (Table 1).

The sera from all vaccinated animals on day 28 post-immunization, with the exception of the placebo recipients, contained serum HAI and neutralizing antibodies (Tables 4 and 5). Vaccination of monkeys with r-bPIV-3 resulted in a HAI titre of 64·0 on day 21 when tested with bPIV-3 antigen (Table 4). When the same serum was tested with a heterologous hPIV-3 antigen, the HAI titre was 11·3. At 1 week later (day 28), the heterologous HAI titre was comparable to the day 21 titre (i.e. 13·5). r-bPIV-3 elicited a similar HAI antibody response to both homologous and heterologous antigens as biological bPIV-3 (Tables 2 and 4). Interestingly, the HAI titres from day 21 sera of animals
immunized with b/hPIV-3 were 76.1 for the hPIV-3 antigen and 11.3 for the bPIV-3 antigen. The HAI titres for hPIV-3 were 215.5 for the homologous hPIV-3 antigen and 22.6 when using bPIV-3 antigen (Table 4). The neutralizing antibody response induced by r-bPIV-3 and b/hPIV-3 on day 28 was measured on both bPIV-3 and hPIV-3 substrates. r-bPIV-3 induced a neutralizing antibody titre of 107.6 and 9.5 for bPIV-3 and hPIV-3 antigens, respectively. These titres were similar to those observed for biological bPIV-3. b/hPIV-3 elicited GMTs of 26.9 and 90.5 for bPIV-3 and hPIV-3 substrates, respectively. hPIV-3 displayed neutralizing antibody titres of 38.1 for the bPIV-3 substrate and 304.4 for the hPIV-3 antigen (Table 5).

**r-bPIV-3- or b/hPIV-3-immunized rhesus monkeys were protected from hPIV-3 challenge (Study B)**

To demonstrate that r-bPIV-3 and b/hPIV-3 were efficacious, an hPIV-3 challenge was carried out in rhesus monkeys. In contrast to Study A, in Study B only a single vaccine dose was administered to the animals prior to hPIV-3 challenge. Placebo recipients challenged with hPIV-3 shed 10^4.8 and 10^3.3 TCID50 ml^-1 in the nasopharynx and trachea, respectively (Table 1). The animals that had received r-bPIV-3, b/hPIV-3 or hPIV-3 displayed greatly reduced peak titres in both the LRT and URT (Table 1). For r-bPIV-3, 10^0.5 and 10^0.3 TCID50 hPIV-3 ml^-1 was observed post-challenge. Interestingly, shed challenge virus was not detected in samples derived from b/hPIV-3-vaccinated primates, even after haemadsorption of the infected monolayers with gp red blood cells. Monkeys that were immunized with hPIV-3/JS shed 10^1.2 TCID50 ml^-1 of challenge virus in the nasopharynx. The titre observed in the nasopharynx was due primarily to a single animal in that group that did not shed high titres of virus or develop a robust immune response following primary immunization.

Following hPIV-3 challenge, the serum HAI titres were boosted in all animals, independent of the vaccine received on day 0 (Table 4). The monkeys that were vaccinated with r-bPIV-3 increased HAI titres to both homologous bPIV-3 and heterologous hPIV-3 antigens 3-fold and 23-fold, respectively, over the titres observed on day 21. The animals immunized with b/hPIV-3 increased HAI titres for the hPIV-3 antigen 4-fold and to the bPIV-3 antigen 2-fold. The hPIV-3-vaccinated animals also increased the hPIV-3 HAI titre more so than for the bPIV-3 antigen. The neutralizing antibody response was also stimulated

**Table 4. Serum HAI antibody response upon immunization of rhesus monkeys with r-bPIV-3, b/hPIV-3 and hPIV-3 (Study B)**

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>No. of animals</th>
<th>Serum HAI GMT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 (pre-dose)</td>
<td>Day 21 (post-dose)</td>
</tr>
<tr>
<td>r-bPIV-3</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>b/hPIV-3</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>hPIV-3/JS</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>Placebo</td>
<td>4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Day 0, pre-dose; day 21, 3 weeks post-dose; day 28, pre-challenge; day 56, 4 weeks post-challenge.
†Antigen used in the HAI assay.

**Table 5. Serum neutralizing antibody response upon immunization of rhesus monkeys with r-bPIV-3, b/hPIV-3 and hPIV-3 (Study B)**

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>No. of animals</th>
<th>Serum neutralizing GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bPIV-3 (grown in Vero cells)*</td>
<td>hPIV-3 (grown in LLC-MK2 cells)*</td>
</tr>
<tr>
<td></td>
<td>Day 0†</td>
<td>Day 28†</td>
</tr>
<tr>
<td>r-bPIV-3</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>b/hPIV-3</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>hPIV-3/JS</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>Placebo</td>
<td>4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Antigen used in the neutralization assay.
†Day 0, pre-dose; day 28, pre-challenge; day 56, 4 weeks post-challenge.
DISCUSSION

The goal of the two primate studies described here was to demonstrate comparability of bPIV-3 vaccine manufactured in FRhL-2 and Vero cells with respect to attenuation, immunogenicity and efficacy, and to show that the plasmid-derived bPIV-3 and the chimeric b/hPIV-3 maintained the attenuation phenotype and protected primates from hPIV-3 challenge. The observation that the bPIV-3 vaccine was attenuated in rhesus monkeys and humans suggested that bPIV-3 could be used as a hPIV-3 vaccine or vector for delivery of foreign antigens (van Wyke Coelingh et al., 1988; Karron et al., 1995). This concept was supported by data obtained from clinical trials showing that a humoral immune response to hPIV-3 was elicited in children upon bPIV-3 vaccination (Karron et al., 1995; Lee et al., 2001).

While the magnitude of the antibody responses in children following bPIV-3 vaccination suggested that they would be protected from hPIV-3, there are no data to support this conclusion. Therefore, we sought to demonstrate in a primate challenge model that vaccination with bPIV-3, r-bPIV-3 or b/hPIV-3 could protect animals from challenge with hPIV-3. Comparison of the results obtained in Study A and Study B demonstrated that r-bPIV-3 grown in SF Vero cells was as attenuated and efficacious as biological bPIV-3 produced in FRhL-2 or Vero cells cultured with FBS. Thus, FBS in the culture medium did not affect the immunogenicity of the various PIV-3 vaccines.

Two vaccines, bPIV-3(F) and bPIV-3(V), were compared directly for replication in the respiratory tract of rhesus monkeys (Study A). Virus shedding data obtained after the first dose of bPIV-3(F) were similar to results reported previously (van Wyke Coelingh et al., 1988) and confirmed that this vaccine was attenuated compared to hPIV-3. Similarly, virus shedding results obtained after the first dose of bPIV-3(V) demonstrated that the vaccine was attenuated relative to hPIV-3. A quantitative analysis of the virus shedding results described above demonstrated that bPIV-3 vaccines, regardless of whether produced in FRhL-2 or Vero cells, were attenuated in the URT and LRT of rhesus monkeys in comparison to hPIV-3. Study B showed that r-bPIV-3, derived from cDNA, was attenuated compared to hPIV-3 and was as attenuated as biological bPIV-3 in the respiratory tract of rhesus monkeys. Both recombinant and biological bPIV-3 showed titre reductions of 25- and 63-fold in the URT, respectively, and 79-fold reductions in the LRT. b/hPIV-3 displayed an intermediate attenuation phenotype between the attenuated bPIV-3 and the non-attenuated hPIV-3, which indicated that some attenuation determinants are located in the F and HN genes, as suggested previously (Schmidt et al., 2000). Whether this decrease of attenuation of b/hPIV-3 in rhesus monkeys will affect its safety profile in humans and whether insertion of additional viral genes encoding protective antigens from other respiratory viruses will compensate for the decreased attenuation of this vector remains to be determined.

Serological data obtained after the first dose confirmed that the bPIV-3 vaccines prepared in FRhL-2 and Vero cells were antigenically comparable and that immunogenicity was independent of the presence or absence of FBS in the Vero cell medium. The serological responses obtained from r-bPIV-3 were also similar to those of biological bPIV-3. All three serological parameters examined (HAI response, neutralizing response and IgA) showed that the humoral immune responses to the homologous bPIV-3 as well as to the heterologous hPIV-3 were within 2-fold of each other. Titres within 4-fold of each other in this type of serological assays are considered comparable. Interestingly, b/hPIV-3 elicited an immune response that resembled hPIV-3 more closely than bPIV-3. Humanizing the surface glycoproteins of bPIV-3 may have created a virus that elicits an immune response very similar to hPIV-3 infections.

In Study A, virus shedding was greatly reduced after administration of the second dose. While no shedding was observed in animals re-vaccinated with hPIV-3(V), hPIV-3(F) or bPIV-3(F), in one animal re-vaccinated with bPIV-3(V), a low level of virus shedding was observed. This suggested that even one dose of bPIV-3 vaccine can induce an immune response that can prevent, or significantly reduce, a secondary infection. This hypothesis was tested in Study B, in which the animals were challenged after receiving only a single vaccination dose. Here, the animals were protected effectively from hPIV-3 challenge. Animals that were vaccinated with r-bPIV-3 or b/hPIV-3 displayed no or low levels of virus shedding, similar to hPIV-3-vaccinated animals. The similarity in the virus shedding data obtained after the second dose with bPIV-3 vaccine grown in either Vero or FRhL-2 cells also demonstrated that the vaccines produced in the two cell substrates are equivalent. This observation was supported by the serological data that showed that while the serum HAI response to both homologous and heterologous viruses increased after the second dose of the two vaccines, the titres evoked were not different.

Both bPIV-3(F) and bPIV-3(V) vaccines provided complete protection from hPIV-3 in the URT and nearly complete protection in the LRT. The serological data showed that following the hPIV-3 challenge, a 2-fold increase in the HAI response against bPIV-3 antigen was induced in animals immunized with bPIV-3(F) or bPIV-3(V) and against hPIV-3 antigen in animals immunized with hPIV-3(F) or hPIV-3(V). In contrast, animals immunized with
bPIV-3(F) or bPIV-3(V) showed a 10-fold increase in HAI titre against hPIV-3 antigen after the animals were challenged with bPIV-3. This indicated that the bPIV-3(F) and bPIV-3(V) vaccines were capable of priming the immune system in rhesus monkeys so that the humoral response could be efficiently boosted when the animals encounter hPIV-3 antigen at a later time. This study focused on induction of serum HAI and neutralizing antibodies because of their established central role in protection from PIV-3 infection and disease in humans. Although homologous antibody titres were higher than heterologous titres, the high level of heterologous protection observed in this study is not unexpected because of the multiple HN and F neutralization epitopes that are shared by bPIV-3 and hPIV-3. The epitopes responsible for the generation of T-cell responses by the HN, F, and other ‘internal’ proteins, which are highly conserved between the bovine and human viruses, are currently unknown. However, based on the importance of cell-mediated immunity for humans to recover from paramyxovirus infections, it is likely that T-cell responses to bPIV-3 also contributed to the high level of heterologous protection observed after hPIV-3 challenge.

The primate studies described here showed that rhesus monkeys can be used as a challenge model to demonstrate efficacy of PIV-3 vaccines. While we cannot be certain that these vaccines will be efficacious in humans, previous studies have demonstrated a good correlation between the efficacy of bPIV-3 vaccines in monkeys and humans (van Wyke Coelingh et al., 1988; Karron et al., 1995, 1996) and none of the data obtained in this study would predict that these vaccines do not protect humans. The monkey model was employed here to show several points: (i) bPIV-3 vaccines prepared in Vero cells are equivalent to bPIV-3 vaccines grown in FRhL-2 cells. However, manufacturing bPIV-3 vaccines in Vero cells offers several commercial advantages over preparing bPIV-3 in FRhL-2 cells. For example, Vero cells grow faster than FRhL-2 cells and can be cultured for a greater number of passages. The peak titre of bPIV-3 is 30- to 50-fold higher in Vero than in FRhL-2 cells and is reached earlier. The combination of the shorter cycle time and higher titre render bPIV-3 vaccines derived from Vero cells preferable from a commercial standpoint; (ii) a recombinant bPIV-3 produced in SF Vero cells is an attenuated and efficacious as biological bPIV-3 produced in Vero cells grown in media containing FBS; (iii) b/hPIV-3 appears to display an intermediate attenuation phenotype between bPIV-3 and hPIV-3, protected completely from hPIV-3 challenge and stimulated high hPIV-3 antibody titres. Some genetic determinants responsible for the attenuation phenotype appear to be located in the bPIV-3 F and HN genes, which will be confirmed by performing additional primate studies. Nevertheless, b/hPIV-3 represents a promising vaccine vector to express antigens derived from other virus pathogens because additional gene insertions into the b/hPIV-3 genome are expected to attenuate virus replication in primates and humans. Thus, a balance should be achieved between virus replication, antigen expression and induction of a protective immune response in vaccinees.

**ACKNOWLEDGEMENTS**

We thank Ken Draper at Sierra Biomedical and Richard Bradbury at BioQual for expertise with the primate studies. We are grateful to Richard Spaete and Harry Greenberg for critical reading and comments on the manuscript.

**REFERENCES**


