Bovine parainfluenza virus type 3 (PIV3) expressing the respiratory syncytial virus (RSV) attachment and fusion proteins protects hamsters from challenge with human PIV3 and RSV

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Parainfluenza virus type 3 (PIV3) and respiratory syncytial virus (RSV) are the main causes of ubiquitous acute respiratory diseases of infancy and early childhood, causing 20–25 % of pneumonia and 45–50 % of bronchiolitis in hospitalized children. The primary goal of this study was to create an effective and safe RSV vaccine based on utilizing attenuated bovine PIV3 (bPIV3) as a virus vector backbone. bPIV3 had been evaluated in human clinical trials and was shown to be attenuated and immunogenic in children as young as 2 months of age. The ability of bPIV3 to function as a virus vaccine vector was explored further by introducing the RSV attachment (G) and fusion (F) genes into the bPIV3 RNA genome. The resulting virus, bPIV3/RSV(I), contained an insert of 2900 nt, comprising two translationally competent transcription units. Despite this increase in genetic material, the virus replicated to high titres in Vero cells. This recombinant virus expressed the RSV G and F proteins sufficiently to evoke a protective immune response in hamsters upon challenge with RSV or human PIV3 and to elicit RSV neutralizing and PIV3 haemagglutinin inhibition serum antibodies. In effect, a bivalent vaccine was produced that could protect vaccinees from RSV as well as PIV3. Such a vaccine would vastly reduce the respiratory disease burden, the associated hospitalization costs and, most importantly, decrease morbidity and mortality of infants, immunocompromised individuals and the elderly.

INTRODUCTION

Human respiratory syncytial virus (RSV) has been recognized as a worldwide pathogen of great importance for the past four decades. In the United States alone, ~100 000 infants are hospitalized annually with RSV infections (Hall, 2001). RSV represents a health threat for infants, the elderly and immunocompromised individuals (Hall, 1999; Falsye et al., 1995). Protection against disease following RSV infection has been attributed to both secretory antibodies and cellular immunity (Chanock & Murphy, 1991). Therefore, effective vaccines for RSV should stimulate mucosal and cellular immune responses. Live attenuated vaccines administered intranasally, mimicking 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.

Recently, a live, attenuated, cold-passaged (cp), temperature-sensitive (ts), intranasally administered RSV candidate vaccine (cpts-248/404) was evaluated in adults and children for safety, infectivity and immunogenicity (Crowe et al., 1994a, b; Karron et al., 1997). cpts-248/404 did not cause fever or lower respiratory tract illness and was not transmitted from infant to infant. However, nasal congestion, which occasionally led to difficulty in eating and irritability, was judged sufficiently disruptive to the infant and family to make this an undesirable vaccine (Karron et al., 1997). Based on these experiences with live attenuated vaccines, new strategies are needed to produce a RSV vaccine that will effectively protect infants against RSV infection, yet will not result in upper respiratory tract congestion.

In this study, we wanted to determine whether bovine parainfluenza virus type 3 (bPIV3) would tolerate the insertion of two translationally active transcription units in its genome as well as generate a novel bPIV3-vectored RSV vaccine candidate. bPIV3 constitutes a promising virus vector, which was shown in human clinical trials to be attenuated, immunogenic, non-transmissible in a day care centre setting and genetically stable in children as young as 2 to 6 months of age (Karron et al., 1996). The capacity of the bPIV3 vaccine to replicate in the nasal cavity without causing respiratory illness demonstrated its attenuation in young seronegative children (Karron et al., 1996). Recombinant bPIV3 (r-bPIV3) has been used successfully as a virus vector to express the highly conserved human
PIV3 (hPIV3) fusion (F) and haemagglutinin–neuraminidase (HN) genes (Haller et al., 2000; Schmidt et al., 2000). To determine whether bPIV3 could be used to express heterologous antigens in addition to bPIV3 antigens, the G and F genes of RSV were selected as they represent the major antigens responsible for eliciting neutralizing antibodies. The safety and attenuation profile of bPIV3 in infants support its use as a vector to deliver RSV antigens to this population. The r-bPIV3 expressing RSV surface glycoproteins is expected to elicit production of RSV serum antibodies and cell-mediated immune responses, yet not cause disease in infants.

The chimeric bPIV3 virus characterized in this study contained the RSV G and F genes inserted downstream of the HN gene of bPIV3. The recombinant bPIV3 harbouring the RSV surface glycoproteins, bPIV3/RSV(I), was shown by biochemical and immunological assays to express the RSV G and F proteins. Studies in small animals showed that bPIV3/RSV(I) was restricted for replication in hamsters, yet bPIV3/RSV(I)-immunized animals were protected from challenge with either hPIV3 or RSV. The bPIV3/RSV(I) construct validated the use of bPIV3 as a virus vector. The insertion of two foreign transcription units was tolerated and both genes were expressed. The recombinant bPIV3/RSV(I) described here will be evaluated further for safety and efficacy in primates as a RSV vaccine candidate.

**METHODS**

**Viruses and cells.** RSV strain A2, r-bPIV3 and bPIV3/RSV(I) were grown in Vero cells in Opti-MEM (Gibco-BRL). Modified vaccinia virus Ankara, which expresses the phage T7 RNA polymerase, was grown in chicken embryonic kidney cells (SPAFAS). Vero, HeLa and Hep-2 cells were maintained in MEM (JRH Biosciences) supplemented with 10 % FBS, 2 mM l-glutamine, non-essential amino acids and antibiotics. BHK-21 cells were grown in Glasgow medium (Gibco-BRL) supplemented with 5 % FBS, 2 mM l-glutamine, 10 % tryptose phosphate broth, 10 mM HEPES and antibiotics.

**Construction of full-length bPIV3/RSV(R) and bPIV3/RSV(I) cDNAs.** Oligonucleotides used to amplify by PCR the RSV G and F genes from RSV-infected Vero cells were complementary to the 5' end of the RSV G gene and encoded an NheI restriction enzyme site or complementary to the 3' end of the RSV F gene and encoded a SalI site. The 2·8 kb PCR fragment was digested with NheI/SalI and cloned into pGEM3 (Promega). The RSV G and F genes were sequenced to confirm the presence of open reading frames. A bPIV3 gene end gene start sequence was introduced into the intergenic sequence between the RSV G and F genes using QuickChange mutagenesis (Stratagene). The plasmid bPIV3/NS, which contains the bPIV3 genome, described previously (Haller et al., 2001), was cleaved with NheI (nt 5042)/SalI (nt 8530) to remove the bPIV3 F and HN genes, and the RSV G and F genes were inserted to yield the full-length cDNA of bPIV3/RSV(R). The M–G and F–I intergenic junctions of this plasmid were sequenced.

To generate a cDNA for bPIV3/RSV(I), a DNA fragment harbouring the RSV G and F genes flanked by SalI restriction enzyme sites was generated by PCR and cloned into pGEM3. bPIV3 gene end/gene start sequences were introduced upstream of the RSV G and the RSV F genes by PCR. This plasmid was digested with SalI, resulting in a 2·8 kb DNA fragment. The cDNA containing bPIV3/N/S was linearized with SalI (nt 8530) and the RSV G and F gene fragment was inserted to construct the plasmid bPIV3/RSV(I). The H–G and the RSV F–bPIV3 L gene junctions were sequenced. Both cDNA constructs were designed to obey the 'rule of six'.

**Transfection of full-length viral cDNA-containing plasmids.** Transfections of viral cDNAs were carried out as described previously (Haller et al., 2000). The presence of bPIV3/RSV(R) or bPIV3/RSV(I) was confirmed by immunostaining of virus-infected monolayers using RSV antiserum (Biotest). Following three cycles of plaque purification at 33 °C, virus stocks were prepared in Vero cells.

**Growth curves.** Vero or BHK-21 cells were grown to 90% confluency and infected at a m.o.i. of 0·1 with r-bPIV3, bPIV3/RSV(I) or RSV. Infected monolayers were incubated at 33, 37, 39 and 40 °C. At 0, 24, 48, 72, 96 or 120 h post-infection (p.i.), the cells and media were harvested. Virus titres for each time point were determined by plaque assay on Vero cells that were immunostained using RSV antiserum.

**Immunoblot and immunoprecipitation analyses.** For Western blot analysis, Vero cells were infected with r-bPIV3, bPIV3/RSV(I) or RSV at an m.o.i. of 0·1. At 48 h p.i., proteins were extracted with lysis buffer. The cell lysate was fractionated on a 10% protein gel, transferred onto a nylon membrane and probed with RSV G68 mAb (Martinez et al., 1997). Protein–antibody complexes were visualized by chemiluminescence (Amershams).

For immunoprecipitation, Vero cells were infected with r-bPIV3, bPIV3/RSV(I) or RSV at an m.o.i. of 1·0. At 24 h p.i., the cells were removed and 0·5 ml DME lacking cysteine and methionine but containing 10 % FBS were washed once with cysteine- and methionine-free DME (ICN) and incubated in the same media for 30 min. The medium was then removed and added 0·5 ml DME lacking cysteine and methionine but containing 10 % FBS. After 5 h at 37 °C, medium was removed and infected cells were lysed in 0·3 M RIPA buffer containing protease inhibitors. The cell lysate was incubated with RSV F 1200 mAb (Beeler & van Wyke Coelingh, 1989) and bound to anti-mouse IgG–agarose (Sigma). After washing three times with 0·5 M RIPA buffer, samples were fractionated on a 12% protein gel. The gel was dried and exposed to MR-X film.

**Flow cytometric analysis.** Vero cells were infected with r-bPIV3, bPIV3/RSV(I) or RSV at an m.o.i. of 0·1 or were mock infected. Infected monolayers were incubated for 24 h, trypsinized using Versene (Gibco-BRL), washed with PBS containing 1·0% BSA and incubated with the primary bPIV3 (VMRD), RSV G68 or RSV F 1200 antisera. After 30 min of incubation, cells were washed and incubated with the secondary sheep anti-mouse FITC-conjugated (Biodesign) or rabbit anti-goat PE-conjugated (Sigma) antisera for 30 min. Cells were then washed twice and analysed using a FACS CALIBUR flow cytometer (BD Immunocytometry Systems).

**Small animal studies.** Hamsters (5-week-old, 8–12 animals per group) were infected intranasally with 1·0 x 10⁶ p.f.u. r-bPIV3, bPIV3/RSV(I), RSV or placebo (Opti-MEM) in a 0·1 ml volume. The four different groups were maintained separately in microisolator cages. At 4 days p.i., the nasal turbinate and lungs of the animals were harvested and homogenized. The titre of virus present in the tissues was determined by plaque assay on Vero cells that were immunostained with RSV or bPIV3 polyclonal antiserum. For challenge studies, animals were inoculated on day 21 intranasally with 1·0 x 10⁶ p.f.u. of hPIV3 or RSV. At 4 days post-challenge, the nasal turbinate and lungs of the animals were assayed by plaque assay on Vero cells for quantification.
Neutralization assay. Microneutralization assays were performed for bPIV3, bPIV3/RSV(I) or RSV using Vero cells. Antibodies [RSV (Biogenesis), bPIV3 (VMRD) polyclonal antisera or RSV F 1153, RSV F 1243 (Beeler & van Wyke Coelingh, 1989) or Synagis (MedImmune) monoclonal antisera] were used at serial twofold dilutions; the starting dilution was 1:4. Samples were then incubated with 100 p.f.u. bPIV3, bPIV3/RSV(I) or RSV at 4 °C for 60 min. Following incubation, virus/serum mixtures were transferred to 96-well plates of Vero cell monolayers, overlaid with media and incubated at 35 °C for 6 days. Neutralization titres were expressed as the reciprocal of the highest serum dilution that inhibited CPE.

Plaque-reduction neutralization assays were carried out for serum obtained on day 21 p.i. from hamsters immunized with r-bPIV3, bPIV3/RSV(I) or RSV. Hamster sera were serially twofold diluted and incubated with 100 p.f.u. RSV in the presence of guinea pig complement for 1 h at 4 °C. Then the virus/serum mixtures were transferred to Vero cell monolayers and overlaid with methylcellulose. After 5 days of incubation at 35 °C, monolayers were immunostained using RSV polyclonal antiserum for quantification. Neutralization titres were expressed as the reciprocal \( \log_2 \) of the highest serum dilution that inhibited 50% of virus titres.

Haemagglutination inhibition assay. Haemagglutination-inhibition (HAI) assays were performed by incubating serial twofold dilutions of hamster serum with hPIV3 at 25 °C for 30 min. Guinea pig erythrocytes were added to each well and incubation was continued for 90 min to allow haemagglutination to occur. HAI titres were expressed as the reciprocal \( \log_2 \) of the highest serum dilution that resulted in inhibition of red blood cell agglutination.

RESULTS

Generation and recovery of bPIV3/RSV(R) and bPIV3/RSV(I) by reverse genetics

Two approaches were pursued to obtain a recombinant bPIV3 that contained the RSV attachment (G) and fusion (F) genes. First, the bPIV3 F and HN genes were replaced with the RSV G and F genes in order to determine whether the RSV proteins can functionally substitute for the bPIV3 F and HN proteins and mediate virus attachment and entry. The RSV G and F proteins display only a low degree of relatedness to the bPIV3 HN and F proteins (less than 20% identity) at the amino acid level (Fig. 1). Repeated attempts to recover virus from the bPIV3/RSV(R) cDNA were not successful. Second, the RSV G and F gene sequences were inserted into the full-length bPIV3 genome in the HN–L intergenic region (Fig. 1) and infectious virus, bPIV3/RSV(I), was rescued. The recovery of bPIV3/RSV(I) by reverse genetics was further confirmed by immunostaining infected cell monolayers with RSV and bPIV3 polyclonal antisera, revealing giant syncytia (data not shown).

bPIV3/RSV(I) displayed a ts replication phenotype in vitro

To assess the effect of introducing two RSV transcription units into the bPIV3 genome on virus replication, the
kinetics of bPIV3/RSV(I) replication was studied. At 48 h p.i., bPIV3/RSV(I) replicated to $8 \times 10^8$ p.f.u. ml$^{-1}$ at 33°C (Fig. 2A). Lower peak titres of bPIV3/RSV(I) were observed at 37°C (a 0·6 log$_{10}$ reduction) and at 39°C (a 1·6 log$_{10}$ reduction) compared to 33°C (Fig. 2B, C). bPIV3/RSV(I) displayed a delayed onset of virus replication at 39°C. This defect in virus replication was even more exacerbated at 40°C, where peak titres of $5 \times 10^5$ p.f.u. ml$^{-1}$ were obtained 72 h p.i. (Fig. 2D). As was observed previously, r-bPIV3 replicated to 9 log$_{10}$ p.f.u. ml$^{-1}$ at 33°C at 72 h p.i. (Fig. 2A) (Haller et al., 2001). Peak titres of r-bPIV3 remained at similar levels, with only a slight drop of 1·3 log$_{10}$ at 40°C (Fig. 2B–D). As expected, RSV replicated to peak titres of $6 \times 10^4$–$7 \times 10^5$ p.f.u. ml$^{-1}$ at all temperatures tested (Fig. 2). These data showed that bPIV3/RSV(I) was temperature sensitive for growth in Vero cells at 40°C.

Surface glycoproteins of RSV and bPIV3 were expressed by bPIV3/RSV(I)

To demonstrate RSV G protein expression, immunoblots of bPIV3/RSV(I)-, r-bPIV3- and RSV-infected cell lysates were probed with RSV G antiserum (Fig. 3A). RSV G, a 96 kDa glycosylated protein, was expressed by RSV and bPIV3/RSV(I) (Fig. 3A, lanes 1 and 3). The level of RSV G expression was reduced in bPIV3/RSV(I) compared to RSV. No product was observed for lysates derived from r-bPIV3 or mock-infected cells (Fig. 3A, lanes 5 and 6). RSV F protein expression was evident by immunoprecipitation of $^{35}$S-labelled bPIV3/RSV(I)-infected cell lysates using RSV F antisera (Fig. 3B). A band of approximately 45 kDa, the predicted size of the RSV F$_1$ protein, was observed in RSV- and bPIV3/RSV(I)-infected cell lysates (Fig. 3B, lanes 2 and 3). No protein was immunoprecipitated from r-bPIV3-infected cell lysates (Fig. 3B, lane 1). These data demonstrated that bPIV3/RSV(I) expressed both RSV G and F proteins. The F protein precursor, F$_0$, was efficiently and correctly processed by cellular proteases, as was shown for wt RSV F$_0$ protein.

Immunoprecipitations of $^{35}$S-labelled r-bPIV3- or bPIV3/RSV(I)-infected cell lysates with bPIV3 antiserum showed that the levels of the bPIV3 F and HN proteins produced by bPIV3/RSV(I) remained unchanged compared to the protein levels observed for r-bPIV3 (data not shown).

Since the RSV F and G proteins are not essential for replication of bPIV3/RSV(I), it was necessary to determine whether a mixed virus population was present in these virus stocks, i.e. bPIV3 expressing only RSV G or bPIV3 expressing only RSV F, as well as bPIV3 expressing both RSV G and F. The virus stocks characterized in this study were passaged five times in Vero cells. Flow cytometry was used to assess the presence of both RSV and PIV3 antigens in bPIV3/RSV(I)-infected cells (Fig. 4). As a control, r-bPIV3-infected cells were incubated with bPIV3-specific primary antiserum or non-specific goat isotype antiserum and both were labelled with PE. Only the peak representing cells labelled with bPIV3 antiserum shifted to the right.
(Fig. 4B). bPIV3/RSV(I)-infected cell populations expressing RSV F and G proteins or mock-infected cells were also probed with RSV mAbs labelled with FITC. bPIV3/RSV(I)-infected cells displaying RSV proteins exclusively displayed a distinct peak apart from the mock-infected cell population (Fig. 4B). A double-labelling experiment showed that bPIV3/RSV(I)-infected cells incubated with both bPIV3 PE-conjugated and either RSV F or G FITC-conjugated antisera sorted to the upper right quadrant (Fig. 4C). In contrast, mock-infected cells incubated with bPIV3- and RSV-specific antisera were observed solely in the lower left quadrant (Fig. 4C). These results showed that bPIV3/RSV(I) expressed the surface glycoproteins of both bPIV3 and RSV (G and F). A spurious deletion of the RSV G or F genes would have resulted in cell populations expressing solely bPIV3 proteins, which should be observed in the upper left quadrant. If cells were infected with viruses that only expressed RSV proteins, a separate cell population in the lower right quadrant would be expected, but this was not seen in these studies (Fig. 4C). The results obtained from the flow cytometric analysis confirmed that bPIV3/RSV(I)-infected cells expressed both bPIV3 and RSV surface glycoproteins.

bPIV3/RSV(I) was not neutralized by RSV antisera

An important question regarding vaccine safety is whether the RSV antigens expressed by bPIV3 are incorporated into the virion envelope and whether the RSV proteins are functional. To address this question, neutralization assays were performed using bPIV3 and RSV polyclonal antisera as well as three RSV F mAbs (1153, 1243 and Synagis). The results showed that bPIV3/RSV(I) was readily neutralized with the bPIV3 antiserum at a 1:1024 dilution (Table 1) but neutralization was not observed with the RSV polyclonal or monoclonal antisera, even at a 1:4 dilution. The control viruses, r-bPIV3 and RSV, were neutralized with the bPIV3 or RSV polyclonal antisera and the RSV mAbs, respectively (Table 1). These results suggested that the RSV proteins were either not incorporated into the virion envelope or that the amount of RSV proteins present on the virion surface was not sufficient to prevent cell entry upon RSV antibody binding. These studies further indicate that RSV G and RSV F proteins cannot substitute functionally for bPIV3 F and HN mediating virus attachment and cell entry. In the presence of bPIV3 antiserum, bPIV3/RSV(I) can no longer enter the cell and the presence of the RSV surface glycoproteins cannot alleviate this block in virus replication. This observation provides experimental support that a change in tissue tropism is unlikely for bPIV3/RSV(I).

bPIV3/RSV(I) replication was attenuated in hamsters

Syrian golden hamsters are permissive to PIV infections and can serve as a small animal model to study infectivity, replication and immunogenicity of recombinant PIV (Haller et al., 2000; Durbin et al., 1999). It was expected that the
bPIV3/RSV(I) infected and replicated in hamsters, similar to r-bPIV3, since virus neutralization was not observed (Table 1). Surprisingly, bPIV3/RSV(I) replication was severely restricted in the lungs and nasal turbinates of hamsters. Virus titres of $0 \log_{10}$ and $2 \log_{10} \text{p.f.u. g}^{-1}$ tissue, respectively, were obtained (Table 2). r-bPIV3 replicated to $5 \log_{10} \text{p.f.u. g}^{-1}$ tissue in the nasal turbinates and $4 \log_{10} \text{g}^{-1}$ tissue in the lungs of hamsters (Table 2).

Hamsters are only semi-permissive to infection by RSV and, thus, lower virus titres were observed. RSV yielded titres of $3 \log_{10}$ and $3 \log_{10} \text{p.f.u. g}^{-1}$ tissue in the nasal turbinates and lungs of hamsters, respectively (Table 2). The titres of bPIV3/RSV(I) were reduced by 2 log10 in the lungs of hamsters and by $\sim 1 \log_{10}$ in the nasal turbinates compared to RSV. These data suggested that bPIV3/RSV(I) was attenuated for replication in the upper and lower respiratory tract of hamsters. However, this host restriction may be specific to rodents and replication of PIV3/RSV(I) may not be restricted in primates or humans.

**Table 1.** bPIV3/RSV(I) cannot be neutralized with RSV antisera

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<th>Virus</th>
<th>Reciprocal neutralizing antibody dilutions</th>
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<tr>
<td></td>
<td>RSV pAb</td>
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<tr>
<td>r-bPIV3</td>
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bPIV3/RSV(I) was not temperature sensitive for replication in BHK-21 cells

One reason for the low levels of bPIV3/RSV(I) replication in hamsters could be the presence of a ts phenotype in *vivo* that impairs virus replication at the physiological temperature of hamsters. Thus, an assay to determine whether bPIV3/RSV(I) was capable of replicating in BHK cells at the body temperature of hamsters was carried out. Hamsters have a body temperature of 37°C. A plaque assay was performed on BHK cells using r-bPIV3, bPIV3/RSV(I) and RSV. Plaque assays were incubated at 33, 35, 37 and 39°C. At 4 days p.i., infected cell monolayers were immunostained for quantification. A drop in virus titre of bPIV3/RSV(I) was observed, which showed that bPIV3/RSV(I)-infected cells expressed RSV G and F proteins as well as bPIV3 surface glycoproteins.

**Fig. 4.** Flow cytometric analysis of bPIV3/RSV(I)-infected Vero cells. Vero cells were infected at an m.o.i. of 0.1 and at 24 h.p.i. the cells were trypsinized and probed with bPIV3 goat polyclonal antiserum and RSV G68 or RSV F1200 mAbs. The secondary antibodies specific for goat were linked to PE and the secondary antibodies specific for mouse were linked to FITC. (A) r-bPIV3-infected Vero cells incubated with non-specific goat isotype antiserum (dotted line) or with bPIV3 polyclonal antiserum (solid line), both labelled with PE. The lower panel shows bPIV3/RSV(I)-infected (solid line) and mock-treated (dotted line) Vero cells probed with bPIV3 polyclonal antiserum labelled with PE. (B) RSV-infected Vero cells incubated with non-specific mouse isotype antiserum (dotted line) or with RSV F (thin solid line) or RSV G (thick solid line) mAbs labelled with FITC. The lower panel shows bPIV3/RSV(I)-infected cells incubated with RSV F (thin solid line) and RSV G (thick solid line) mAbs linked to FITC. (C) bPIV3/RSV(I)-infected or mock-treated Vero cells in a double label experiment were probed with both bPIV3 polyclonal antibody labelled with PE and RSV F or RSV G mAbs labelled with FITC. When cells were labelled with both FITC and PE, a shift to the upper right corner was observed, which showed that bPIV3/RSV(I)-infected cells expressed RSV G and F proteins as well as bPIV3 surface glycoproteins.
RSV(I) was not observed at any of the temperatures used for this assay (data not shown). The replication impairment of bPIV3/RSV(I) in hamsters was, therefore, not due to a host cell-specific ts phenotype.

To determine whether the restricted replication phenotype of bPIV3/RSV(I) in hamsters can be mimicked in tissue culture, the replication kinetics of bPIV3/RSV(I), r-bPIV3 and RSV were compared in Vero and BHK-21 cells (Fig. 5). All three viruses, bPIV3/RSV(I), r-bPIV3 and RSV, replicated to peak titres of 8·5, 9·0 and 7·1 log₁₀ p.f.u. ml⁻¹ in Vero cells, respectively (Fig. 5A). Replication of r-bPIV3 was reduced by ~1·0 log₁₀ in BHK-21 cells and a peak titre of 8·0 log₁₀ p.f.u. ml⁻¹ was achieved (Fig. 5B). The peak of r-bPIV3 replication in Vero cells occurred at 48 h p.i. (Fig. 5A). In BHK-21 cells, the peak titre of r-bPIV3 was observed at 96 h p.i. (Fig. 5B). RSV replicated to a low titre of 5·9 log₁₀ p.f.u. ml⁻¹ in BHK-21 cells and displayed unstable virus titres after 48 h p.i. (Fig. 5B). A 1·0 log₁₀ reduction in RSV peak titre in BHK-21 cells compared to titres achieved in Vero cells was observed in this study. bPIV3/RSV(I) replication peaked at 48 h p.i. in Vero cells, while a delayed onset of virus replication in BHK-21 cells was observed (Fig. 5A, B). Peak titres of bPIV3/RSV(I) in Vero and BHK-21 cells were lower by ~1·5 log₁₀ compared to the titres obtained in Vero cells. The data from this study showed that all three viruses, r-bPIV3, bPIV3/RSV(I) and RSV, replicated less efficiently in BHK-21 cells. However, the delayed replication of bPIV3/RSV(I) in BHK-21 cells cannot be correlated directly to the impaired in vivo phenotype observed in hamsters.

**bPIV3/RSV(I)-immunized hamsters were protected from challenge with hPIV3 or RSV A2**

Despite the low level of replication of bPIV3/RSV(I) observed in hamsters, animals immunized with bPIV3/RSV(I) were tested in challenge studies for immune protection from hPIV3 and RSV (Table 2). Groups of hamsters were immunized with r-bPIV3, RSV or bPIV3/RSV(I) or received placebo medium intranasally. At 21 days post-immunization, animals were challenged with either hPIV3 or RSV intranasally. bPIV3/RSV(I)-immunized animals displayed hPIV3 virus titres less than 1·6 and 1·3 log₁₀ p.f.u. g⁻¹ tissue in the nasal turbinates and lungs of the hPIV3-challenged hamsters, respectively (Table 2). These results demonstrated that immunization with bPIV3/RSV(I) resulted in complete immune protection from hPIV3. The same level of immunity was observed for animals immunized with r-bPIV3 (Table 2). Only hamsters that received placebo medium or wt RSV displayed high levels of hPIV3 challenge virus, 4·2 and 4·9 log₁₀ p.f.u. g⁻¹ tissue or 4·9 and 5·6 log₁₀ p.f.u. g⁻¹ tissue, respectively, in the nasal turbinates and lungs (Table 2).

**Table 2.** bPIV3/RSV(I) replicates in Syrian golden hamsters and protects from challenge with hPIV3 and RSV strain A2

<table>
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<tr>
<th>Immunizing virus</th>
<th>Mean virus titre p.i. (log₁₀ p.f.u. g⁻¹ tissue ± SE)</th>
<th>Mean virus titre post-challenge (log₁₀ p.f.u. g⁻¹ tissue ± SE)</th>
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<td>r-bPIV3</td>
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<td>Placebo</td>
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**Fig. 5.** Multicycle growth curves of bPIV3/RSV(I), r-bPIV3 and RSV were carried out in Vero cells (A) and BHK-21 cells (B). Collection times were at 0, 24, 48, 72, 96 and 120 h p.i. Samples were analysed for virus titres by plaque assay on Vero cells.
bPIV3/RSV(I)-immunized hamsters that were given a RSV challenge dose intranasally also showed protection from RSV strain A2. bPIV3/RSV(I)-immunized animals displayed titres of 1·7 and 1·4 log10 p.f.u. g−1 tissue in the nasal turbinates and lungs, respectively (Table 2). Hamsters that were inoculated with RSV initially and then challenged with RSV displayed 1·2 and 1·5 log10 p.f.u. g−1 tissue in the nasal turbinates and lungs (Table 2). Animals that had been immunized with placebo medium or r-bPIV3 and were challenged with RSV showed titres of 3·8 and 3·0 log10 p.f.u. g−1 tissue or 3·3 and 2·9 log10 p.f.u. g−1 tissue, respectively, in the nasal turbinates and lungs (Table 2). bPIV3/RSV(I) immunization reduced RSV virus loads in the upper and lower respiratory tracts of hamsters by 2·1 and 1·6 log10 compared to the RSV-challenged animals that had received placebo medium (Table 2). Immunization with RSV reduced virus titres by 2·6 and 1·5 log10 p.f.u. g−1 tissue in the nasal turbinates and lungs, respectively (Table 2). Therefore, bPIV3/RSV(I) protected the lower respiratory tract of hamsters as well as wt RSV and showed slightly less protection of the upper respiratory tract than RSV-immunized animals. bPIV3- RSV(I) animals were completely protected from challenge with hPIV3.

Hamsters vaccinated with bPIV3/RSV(I) elicited RSV neutralizing and PIV3 HAI serum antibody responses

Hamster sera obtained 21 days post-immunization were analysed for the presence of RSV neutralizing and PIV3 HAI serum antibodies. Despite low levels of replication observed for bPIV3/RSV(I), RSV neutralizing antibodies were detected in the day 21 hamster sera (Table 3). RSV antibody titres of 5·4 log2 were observed for bPIV3/RSV(I) hamster sera, while wt RSV sera contained slightly higher antibody titres of 6·6 log2. HAI antibody titres were also determined for the day 21 hamster sera. Sera obtained from animals vaccinated with bPIV3/RSV(I) had slightly lower HAI titres of 2·2 compared to HAI titres present in bPIV3 hamster sera, which displayed titres of 3·3. In general, HAI titres produced by bPIV3-vaccinated hamsters were low; this has been observed previously (Haller et al., 2000). Thus, bPIV3 and bPIV3/RSV(I) may mediate immune protection by cellular immune pathways.

**DISCUSSION**

The application of live attenuated virus vectors, such as bPIV3, to deliver RSV antigens represents an approach different from generating live, attenuated RSV or RSV subunit vaccines, none of which have been successful. bPIV3 constitutes a promising vaccine vector because it was already evaluated positively for safety and immunogenicity in phase 1 and 2 human clinical trials as hPIV3 vaccine candidate. It is expected that the bPIV3/RSV(I) virus will display all of the desirable restricted replication characteristics in humans, since all of the attenuation determinants were retained in the bPIV3 vaccine genome. This is the advantage bPIV3 has over the related virus vector, a chimeric bovine/human PIV3, which may have lost potential attenuation-determining sequences by substituting the bPIV3 F and HN genes with those of hPIV3 (Schmidt et al., 2000). The safety of RSV vaccines is an important concern, since the vaccine will be administered to infants. The ideal RSV vaccine should elicit an immunity that is at least as effective as wt RSV. The vaccine should induce protective levels of neutralizing antibody as well as CD8+ RSV-specific cytotoxic T cell and CD4 responses like that evoked by wt RSV. Further studies remain to be carried out to determine whether the bPIV3/RSV(I) can elicit the desired immune response in humans.

The recombinant bPIV3 characterized in this study expressed both RSV G and F genes. bPIV3/RSV(I) was temperature sensitive for growth in Vero cells at 40°C; however, it replicated to high virus titres at 33°C. In vivo, bPIV3/RSV(I) was restricted for replication in the respiratory tract of hamsters. Despite lower levels of replication observed for bPIV3/RSV(I) in hamsters, immunized animals were protected from challenge with hPIV3 or RSV A2. The restricted replication of bPIV3/RSV(I) in hamsters could not be mimicked in BHK-21 cells. bPIV3/ RSV(I) did not display a host cell-specific ts phenotype in BHK-21 cells at the body temperature of hamsters (37°C), which could have explained its poor replication in hamsters. The impaired replication of bPIV3/RSV(I) in vivo may be due to inserting two transcription units between the HN and L genes of bPIV3, thereby adding ~2900 nt to the viral genome. Skiadopoulos et al. (2000) studied the effect of increasing the PIV3 genome on...

**Table 3. Vaccination of hamsters with bPIV3/RSV (I) induces serum HAI and neutralizing antibodies**

Hamsters were immunized with the viruses indicated. Neutralizing antibody titres were determined by a 50 % plaque-reduction assay; neutralizing antibody titres and HAI antibody titres of hamster pre-serum were <1·0. ND, Not determined.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Neutralizing antibody response to RSV (mean reciprocal log2 ± SE)</th>
<th>HAI antibody response to hPIV3 (mean reciprocal log2 ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r-bPIV3</td>
<td>ND</td>
<td>3·3 ±1·0</td>
</tr>
<tr>
<td>bPIV3/RSV(I)</td>
<td>5·4 ±0·7</td>
<td>2·2 ±0·9</td>
</tr>
<tr>
<td>RSV strain A2</td>
<td>6·6 ±0·4</td>
<td>ND</td>
</tr>
</tbody>
</table>
virus replication. Non-coding or coding sequences up to ~3900 nt were inserted into a recombinant bPIV3 between the HN and L genes. Viruses harbouring the largest inserts replicated efficiently in vitro. However, viruses containing a genome that was increased by greater than 3000 nt exhibited restricted replication in hamsters. The genes inserted into bPIV3/RSV(I) were not only transcriptionally active but also translationally competent, the latter of which differed from the inserts used by Skiadopoulos and co-workers, which may be the reason for the increased in vivo attenuation observed in this study.

We demonstrated protective immunity of bPIV3/RSV(I)-vaccinated animals not only for RSV but also for hPIV3, thereby expanding the use of bPIV3 for the generation of bivalent paediatric vaccines. bPIV3/RSV(I)-immunized animals produced RSV neutralizing and PIV3 HAI serum antibodies at levels slightly lower than the parental virus strains. There are several advantages of using live, attenuated, intranasally administered vaccines: (1) live virus vaccines stimulate immune responses similar to wt virus infections, inducing both systemic and local immunity (Murphy et al., 1994); (2) maternal antibodies present in infants do not appear to interfere with vaccine virus replication in the nasopharynx and its ability to induce protective immunity (Murphy et al., 1994); (3) live attenuated RSV vaccines are not likely to cause RSV disease potentiation during subsequent, naturally occurring, wt RSV infections in immunized infants (Chanock & Murphy, 1991).

Results obtained by other investigators underscore further the high likelihood of success for bPIV3 as a virus vaccine vector. Recently, Schmidt et al. (2001, 2002) generated recombinant chimeric bovine/human PIV3 expressing RSV G and/or F proteins. Hamsters immunized with these recombinant viruses were protected in challenge studies from RSV as well as bPIV3. The position of the RSV genes introduced into the PIV3 genome may specify a host range phenotype. The recombinant bovine/human PIV3 harboured the RSV genes at the 3’ end of the viral genome, while bPIV3/RSV(I) contained the RSV G and F genes in the centre of the genome, between the HN and L genes of bPIV3. Schmidt et al. (2001, 2002) employed a chimeric bovine/human PIV3 as a vector backbone to enhance PIV3 antigenicity. However, the humanization of the bPIV3 surface glycoproteins may result in the loss of the critical attenuation phenotype observed in humans. The attenuation phenotype associated with bPIV3 is most likely the result of contributions from multiple viral gene products. Therefore, it is important to pursue development of bPIV3 as a vaccine vector since it retains all of the attenuation determinants.

bPIV3/RSV(I) represents a novel, rationally designed, vaccine candidate intended to protect children, the elderly and immunocompromised individuals from disease caused by RSV and hPIV3. While other virus vectors may be developed in the future to deliver RSV antigens, bPIV3 has a number of advantages, especially regarding safety and genetic stability, that warrant its development as a vaccine vector: (1) the greatest advantage of using bPIV3 is its prior experience in human clinical trials, where bPIV3 was shown to be attenuated in infants (Karron et al., 1996); (2) recombinant bPIV3 expressing both RSV G and F proteins has the advantage that only a single vaccine virus needs to be administered to afford immunity; (3) recombinant bPIV3/RSV(I) contains the entire bPIV3 RNA genome and, thus, is expected to retain all of the attenuation determinants; (4) RSV antigens are delivered within the context of a live virus and, thus, immunopotentiation upon infection with wt RSV is not expected to occur; (5) live bPIV3/RSV(I) vaccine should stimulate both systemic and mucosal immunity. The information gained from studying bPIV3/RSV(I) leads further support to developing bPIV3 as a vaccine vector for expression of viral and bacterial antigens.

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