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Genetic stability of RSV-F expression and the restricted growth phenotype of a live attenuated PIV3 vectored RSV vaccine candidate (MEDI-534) following restrictive growth in human lung cells☆

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ABSTRACT

MEDI-534 is the first live, attenuated and vectored respiratory syncytial virus (RSV) vaccine to be evaluated in seronegative children. It consists of a bovine/human parainfluenza virus type 3 (PIV3) backbone with the RSV fusion glycoprotein (RSV-F) expressed from the second position. The PIV3 fusion and hemaglutinin-neuraminidase proteins are human-derived. No small animal appropriately replicates the restrictive growth of bovine PIV3 (bPIV3) based viruses relative to human PIV3 (hPIV3) observed in the respiratory tract of rhesus monkeys and humans, making analysis of the genetic stability of the attenuation phenotype and maintenance of RSV-F expression difficult. Screening of multiple cell-lines identified MRC-5 cells as supporting permissive growth of hPIV3 while restricting bPIV3 and MEDI-534 growth. In MRC-5 cells, the peak titers of MEDI-534 were more than 20-fold lower compared to hPIV3 peak titers. After more than 10 multicycle passages in MRC-5 cells, genetic alterations were detected in MEDI-534 that contributed to a partial loss in restricted growth in MRC-5 cells and a decrease in RSV-F expression. These adaptive mutations did not occur in the RSV-F gene but were found in the polyA sequence upstream of the transgene. MRC-5 adapted MEDI-534 viruses (1) lost some attenuation but did not replicate to the level of hPIV3 in this cell line, (2) did not completely lose RSV-F expression and (3) were able to elicit a protective anti-RSV immune response in hamsters despite lower levels of RSV-F expression. Interestingly analysis of shed MEDI-534 from a recent clinical trial indicates that in some recipients similar mutations arise by day 7 or day 12 post immunization (in press) suggesting that these mutations can arise rapidly in the human host. The utility and limits of MRC-5 cells for characterizing the attenuation and RSV-F expression of MEDI-534 is discussed.

1. Introduction

Multiple approaches to pediatric respiratory syncytial virus (RSV) vaccination have been evaluated in the clinic over the past 30 years, but to date there are no licensed RSV vaccines [1]. MEDI-534, the most clinically advanced live pediatric RSV vaccine candidate [2], is a chimeric bovine/human parainfluenza virus type 3 (PIV3) that expresses the RSV-F antigen (Fig. 1A). All internal genes are derived from bPIV3 but the bovine F and HN surface glycoproteins are substituted with analogs from hPIV3 [3,4], making MEDI-534 a potential bivalent vaccine against RSV and hPIV3.

For a safe and effective RSV vaccine, two key characteristics are required for MEDI-534: (1) maintenance of attenuation phenotype and (2) maintenance of RSV-F expression. Since host range restriction of MEDI-534 is polygenic and attributed to multiple bPIV3 genes [5], a complete reversion to hPIV3 virulence is unlikely. Insertion of the extra RSV-F transcriptional unit likely also contributes to the attenuation phenotype in humans. Since RSV-F expression is not essential for MEDI-534 replication, there is potential to lose its expression during replication under host-range restricted conditions such as the human respiratory tract. It is known that RSV-F expression is stable following multiple passages of MEDI-534 in permissive cell lines such as VEROs and in lungs of permissive animals such as hamsters (unpublished data). However, these biological systems do not effectively model the host range restriction of bPIV3-based vaccines observed in the respiratory tract of rhesus monkeys and young children [6,7], and up until this point no small animal model or cell line had been identified for pressure testing the phenotypic stability of MEDI-534 attenuation and RSV-F expression. Here we demonstrate that bPIV3 based viruses, unlike hPIV3, are attenuated for replication in the MRC-5 human lung cell

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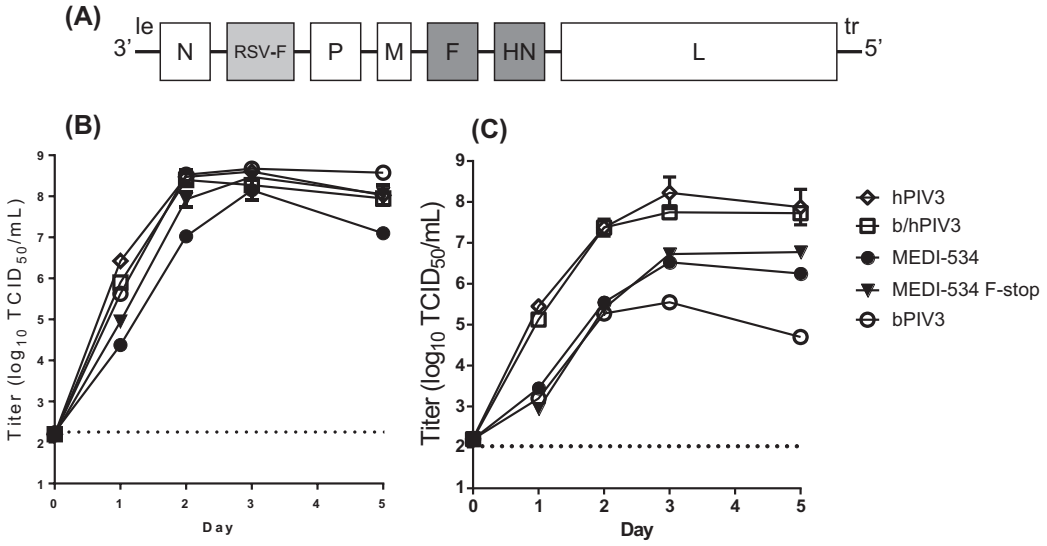

Fig. 1. Growth characteristics of PIV3 based viruses. Schematic diagram of MEDI-534 (A) The RSV F gene (light gray) is in position 2 of the PIV3 genome. Bovine PIV3 genes are shown in white and human PIV3 genes are shown in dark gray. Multicycle growth curves in VERO cells (B) or MRC-5 cells (C). Shown are representative growth curves with the titer for each timepoint determined in quadruplicate. The limit of detection is indicated by dotted lines. Error bars indicate SEM.

line. After multiple passages in this bPIV3 host range restricted system, changes occurred in the upstream polyadenylation signal that reduced RSV-F expression and increased peak replication titers.

A recent Phase 1 study demonstrated that MEDI-534 is safe and immunogenic in healthy RSV and PIV3 seronegative children 6 to <24 months of age [2]. Analysis of shed MEDI-534 viruses from the Phase 1 study identified the presence of variant subpopulations with altered RSV-F expression caused by similar mutations described in this current study (in press), validating the utility of the MRC-5 *in vitro* model to assess the stability of vectored vaccines prior to clinical trials.

2. Materials and methods

2.1. Viruses and cells

MEDI-534 was grown in VERO cells and purified by tangential flow filtration [8]. All other viral stocks were generated as previously described and titered by plaque assay [4,9].

VERO cells were maintained as described previously [4]. MRC-5 cells (passages 19-29; ATCC#CCL-171) were maintained at 37 °C in DMEM supplemented with 10% FBS (Hyclone), 0.1% gentamicin (Gibco), 1% L-glutamine (JRH), and 1% sodium pyruvate (Hyclone).

2.2. Generation of viral cDNA and recombinant viruses

MEDI-534 and its derivatives were engineered and propagated as previously described [4]. For MEDI-534 F-stop, oligonucleotides were designed to convert the starting methionine to a threonine and the 2nd and 28th amino acid to a stop codon using QuikChange (Stratagene). NpolyA virus contains the a1699g mutation in the PIV3-N polyadenylation sequence present in lineages 23A and 23C. M ORF virus contains the S74G amino acid change in the PIV3-M open reading frame (nucleotide change a5862g). The N/M virus contains both mutations, introduced by oligonucleotides (Stratagene QuikChange 2XL). Full-length clones were generated using the 1-5 bPIV3 plasmid subclone and b/hPIV3 genome [9].

Recombinant viruses were generated by transfection of full-length cDNA together with expression plasmids pCite PIV3-N, P and L [3] in BSR-T7 cells as previously described [10].

2.3. Serial passaging of MEDI-534

In three separate infections, MRC-5 cells at 100% confluency were infected with MEDI-534 at an m.o.i. of 0.001. Supernatants were harvested on day 3 post-infection. Virus was stabilized by addition of 10× SPG (10× is 2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, 0.054 M L-glutamate, pH 7.1) prior to freezing at −80 °C. For passages 2-23, an estimated peak titer of ~4.5 $\log_{10}$ $TCID_{50}$/ml for each prior passage day 3 harvest was used to calculate a starting infection m.o.i. of 0.001. Characteristic cytopathic effect was used to confirm the presence of MEDI-534 during passaging. Three separate lineages were maintained in parallel throughout the passaging.

2.4. Growth curves

VERO or MRC-5 monolayers were infected at an m.o.i. of 0.001 and incubated at 37 °C (VERO) or 33 °C (MRC-5) in 5% CO_2 incubators. At indicated time points, supernatants were harvested and stabilized with 1× SPG for storage at −80 °C. Growth curve titers were determined by $TCID_{50}$ [4] in VERO cells. Statistical analysis was by ANOVA, with a Tukey adjustment for pairwise comparisons.

2.5. Western and northern blot

Cell lysates were analyzed by standard western blot. Briefly, VERO cells were infected at an m.o.i. of 0.001 with the indicated viruses and harvested on day 3 post-infection. Western blots were probed with an anti-RSV-F MAb (MedImmune) at a 1:1000 to 1:20K dilution or a rabbit anti-PIV3-N polyclonal antibody (#181, MedImmune) at a 1:40K dilution, followed by HRP conjugated rabbit anti-human IgG or goat anti-rabbit IgG antibody at a 1:2000 to a 1:20K dilution (DAKO). Protein-antibody complexes were visualized by chemiluminescence (ECL-plus, Amersham).

For northern blot analysis, VERO cells were infected at an m.o.i. of 0.001. At 3 days post-infection total RNA was isolated using the QIAGEN RNeasy kit, fractionated on a 1% agarose gel using glyoxal (Ambion) and transferred to a nylon membrane (Schleicher & Schuell). Blots were hybridized with genomic sense transcriptionally labeled (Digoxygen-UTP, Roche) ssRNA probes against RSV-F