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SCIENCE DIRECT
Vaccine 24 (2006) 2674–2684
Vaccine
www.elsevier.com/locate/vaccine

Introducing point and deletion mutations into the P/C gene of human parainfluenza virus type 1 (HPIV1) by reverse genetics generates attenuated and efficacious vaccine candidates

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Received 5 August 2005; received in revised form 21 October 2005; accepted 26 October 2005
Available online 15 November 2005

Abstract

The P/C gene of human parainfluenza virus type 1 (HPIV1) encodes a nested set of related accessory C proteins, C′/C/Y1/Y2, which have been shown in other paramyxoviruses to have a role in evasion of the type I interferon (IFN) response following virus infection. We previously demonstrated that a set of two amino acid substitutions, C^{R84G}/HN^{T553A}, and a separate amino acid substitution, C^{F170S}, are independently attenuating for HPIV1 in African green monkeys (AGMs). However, in each case the attenuation (*att*) phenotype is vulnerable to reversion by a single nucleotide change back to wild type. Using reverse genetics, recombinant HPIV1 (rHPIV1) vaccine candidates were generated that were designed for increased genetic and phenotypic stability by: (i) creating a two-amino acid deletion and substitution at the site of the C^{F170S} mutation, yielding $C^{\Delta 170}$; (ii) introducing a six amino acid deletion in the N-terminal region of C, $C^{\Delta 10–15}$; and (iii) combining these stable deletion mutations with the *att* C^{R84G}/HN^{T553A} mutation. The resulting rHPIV1 vaccine candidates were evaluated for attenuation in hamsters and AGMs and for immunogenicity and protective efficacy in AGMs. The $C^{\Delta 10–15}$ mutation was attenuating in hamsters but not in AGMs, and likely will be of limited value for an HPIV1 vaccine. Conversely, the C^{R84G}/HN^{T553A} mutation set was attenuating in AGMs but not in hamsters. Thus, these two mutations demonstrated reciprocal host range phenotypes involving different regions of C. The $C^{\Delta 170}$ mutation conferred a significant level of attenuation in hamsters and AGMs that closely resembled that of C^{F170S} and will be of particular utility for vaccine development because it involves a deletion of six nucleotides rendering it highly refractory to reversion. The combination of the C^{R84G}/HN^{T553A} mutation set and the $C^{\Delta 170}$ deletion mutation yielded a virus, $rC^{R84G/\Delta 170}HN^{T553A}$, that exhibited a satisfactory level of attenuation in hamsters and AGMs and was immunogenic and highly protective against HPIV1 wt challenge. This virus will be evaluated clinically as a live intranasal HPIV1 vaccine, one that can be further attenuated as necessary by the introduction of additional stabilized *att* mutations previously developed in the L protein.
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Keywords: Human parainfluenza virus; Attenuating mutations; Interferon antagonist; Vaccine candidates; Non-human primate study

1. Introduction

Human parainfluenza virus type 1 (HPIV1) is a member of the *Paramyxoviridae* family of viruses, which encompasses a group of significant human pathogens including respiratory syncytial virus (RSV), HPIV2, HPIV3, measles virus, mumps virus, and the recently identified human metapneumovirus [1,2]. The human parainfluenza viruses belong to the subfamily *Paramyxoviridae* and are further classified into the genera *Respirovirus* (HPIV1 and HPIV3) and *Rubulavirus* (HPIV2). HPIV1, 2 and 3 cause significant respiratory disease in infants and young children, accounting for 6.0, 3.2 and 11.5%, respectively, of pediatric hospitalizations due to respiratory disease, making them collectively the second leading cause of pediatric hospitalizations for respiratory disease

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2005.10.047

behind RSV [1,3]. Parainfluenza viruses are also becoming increasingly recognized as causes of respiratory disease in adults and immunocompromised patients [4–9]. Clinical manifestations range from mild disease, including rhinitis, pharyngitis, and otitis media, to severe disease, including croup, bronchiolitis, and pneumonia [1,10–14]. A licensed vaccine is not available for the prevention of HPIV infection.

HPIV1 is an enveloped, negative sense, non-segmented, single-stranded RNA virus with a genome length of 15,600 nucleotides, containing six genes in the order 3′-N-P/C-M-F-HN-L-5′. Members of the *Paramyxoviridae* subfamily such as HPIV1 conform to a "rule of six", whereby the nucleotide length of the genome must be an even multiple of six in order for efficient replication to occur [15]. HPIV1 encodes three nucleocapsid-associated proteins including the nucleocapsid protein (N), the phosphoprotein (P) and the large polymerase (L); three envelope-associated proteins including the internal matrix protein (M) and the fusion (F) and hemagglutinin-neuraminidase (HN) transmembrane surface glycoproteins; and a nested set of four proteins, C′, C, Y1 and Y2, that are expressed from the C ORF [1]. Each HPIV1 gene encodes a single protein with the exception of the P/C gene, which contains two overlapping open reading frames (ORFs), one coding for P and one coding for the C proteins, which initiate at four separate translational start codons and are carboxy co-terminal. It is unclear whether the Y2 protein is actually expressed in HPIV1 infection, although the sequence of the ORF would be consistent with its expression [16]. One or more forms of C protein are expressed by viruses of the *Respirovirus*, *Morbillivirus*, and *Henipahvirus* genera, but not by viruses of the *Rubulavirus* and *Avulavirus* genera.

The C proteins of HPIV1 are not well characterized. In comparison, the C proteins of murine PIV1 (Sendai virus) have been extensively studied, and Sendai virus is used as a model for HPIV1. Like HPIV1, Sendai virus contains a P gene with two overlapping ORFs coding for the P protein and a nested set of C proteins, C′/C/Y1/Y2. The Sendai P/C gene also encodes additional V and W proteins that are made by RNA editing; however, RNA editing does not seem to occur for HPIV1 and the V ORF is present only as a relict interrupted by stop codons [1]. The Sendai virus C proteins have been identified as accessory proteins, indicating that they are not absolutely essential for virus growth. They are multifunctional proteins that play roles in both the virus replicative cycle and the host response to viral infection, including: (i) inhibiting viral RNA synthesis, which is thought to be mediated by C-L protein binding [17,18]; (ii) promoting viral assembly and/or budding [19,20]; (iii) suppressing apoptosis [21]; and (iv) counteracting the antiviral action of interferon (IFN) [22–27]. Their role in evading the host's innate immune response make the C proteins prime targets for inactivation by mutation since viruses bearing mutations that disable IFN antagonists are attenuated in vivo [28].

The type 1 IFN response is part of the host's innate immune response and also can stimulate the adaptive immune response. The IFN response comprises a three-step cascade of events including: (i) IFN production; (ii) IFN signaling following binding to its receptor; and (iii) an effector phase [28,29]. The type 1 IFN response is triggered following virus infection and induces an antiviral state within infected and neighboring cells. Viruses have evolved mechanisms to circumvent these IFN-induced antiviral mechanisms by inhibiting one or more of these stages in the IFN response [28,30–32]. The V and C accessory proteins of the Paramyxoviruses are potent IFN antagonists [33,34]. The Paramyxovirus V proteins target both IFN production [27,35] and signaling through the IFN receptor/JAK-STAT pathway [36–40]. HPIV1 does not express a V protein and its C proteins are yet to be characterized, but those of its murine counterpart, Sendai virus, have been studied in some detail. The anti-IFN capacity of Sendai virus has been clearly demonstrated [22] and Sendai virus C proteins are known IFN antagonists [23,41,42]. However, the exact mechanisms for C protein-mediated IFN inhibition are not completely defined. Recent studies have determined that Sendai virus C proteins target both IFN production and signaling. IFNβ production is suppressed by Sendai virus C proteins through the inhibition of activation of IFN regulatory factor 3 (IRF-3), a constitutively expressed protein in the cytoplasm of cells that, following activation, translocates to the nucleus to stimulate IFNβ transcription [27]. The C proteins of Sendai virus also interfere with the signaling that occurs following binding of IFN to its receptor, thereby decreasing activation of IFN response genes [43–45]. Alteration of the C proteins, by point mutation or deletion, results in viruses which are attenuated in vitro and in vivo [25,46–49], highlighting the role of the C proteins in viral virulence. Wild type (wt) Sendai virus was rendered avirulent in mice by a point mutation in C, which resulted in a phenylalanine to serine substitution at amino acid position 170 (C^{F170S}) [46–48]. Similarly, a Sendai virus mutant containing a deletion of amino acids 10–15 in C ($C^{\Delta 10-15}$), which expressed mutant C′/C proteins and wt Y1 and Y2 proteins, was attenuated in mice [25,49]. These findings indicate that the C proteins are crucial to virus growth in vivo and are potential targets for developing attenuated viruses. The HPIV1 C proteins are yet to be characterized for their role in IFN inhibition, however, previous studies indicate that they share similarities with Sendai virus, with HPIV1 P/C gene mutants being attenuated in vivo [50,51].

Our laboratory is developing live attenuated intranasal virus vaccines for HPIV1 using reverse genetics [50–52]. We previously reported that the C^{F170S} mutation that attenuated Sendai virus for rodents [46] also attenuated HPIV1 for rodents and African green monkeys (AGMs) when imported into this human virus [50,51]. In addition, we described the novel C^{R84G}/HN^{T553A} mutations, which are a set of two spontaneous amino acid substitutions that together attenuated HPIV1 for AGMs but not hamsters [51]. However, the C^{F170S} mutation and the C^{R84G}/HN^{T553A} set could each be lost by single nucleotide reversion, making the attenuation phenotype potentially unstable. Here, we describe a strategy to develop attenuating C mutations involving