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Genetic stability determinants of temperature sensitive, live attenuated respiratory syncytial virus vaccine candidates

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Abstract

An intranasally delivered, live attenuated, temperature sensitive (*ts*) respiratory syncytial virus vaccine candidate, rA2cp248/404/1030ΔSH, exhibits a low level of genetic instability in clinical studies, in contrast to the relatively high stability of two similar candidates, *cpts*248/404 and rA2cp248/404ΔSH. The latter strains, containing two *ts* mutations (248ts and 404ts), are partially growth restricted at 37 °C, whereas, rA2cp248/404/1030ΔSH contains an additional *ts* mutation (1030ts) that increases attenuation and partially restricts virus growth at 35 °C. Since the maximum human airway temperature is 35.5 °C, we investigated whether growth restriction at 35 °C contributes to genetic instability of rA2cp248/404/1030ΔSH in vitro. We conducted in vitro passage studies with the three strains at 32 °C (a fully permissive growth temperature) and 35 °C (restrictive for only rA2cp248/404/1030ΔSH). Instability of the *ts* phenotype was observed only in rA2cp248/404/1030ΔSH passaged at 35 °C, and corresponded with reversion at the 248ts or 1030ts mutation sites, as observed in clinical studies. This study indicates that *ts* mutations that partially restrict replication at physiologic temperatures may contribute to genetic instability of viruses in vivo. In vitro passage studies performed at appropriate temperatures can be used to assess genetic stability and to prioritize *ts* vaccine candidates for clinical evaluation.

Keywords: Vaccine; Respiratory syncytial virus (RSV); In vitro screening

1. Introduction

Respiratory syncytial virus (RSV) is a non-segmented, negative-sense, single-stranded RNA virus in the *Paramyxoviridae* family and *Pneumovirus* genus (Collins et al., 2001). It is the most important etiologic agent of bronchiolitis and pneumonia in infants and children (Blount et al., 1956; Collins et al., 2001; Dudas and Karron, 1998). Despite the urgent need for preventive measures, no licensed vaccine is available.

A number of intranasally delivered, live attenuated RSV vaccine candidates have been clinically evaluated for safety, immunogenicity, and genetic stability in young children. Clinical studies conducted on cold passaged (*cp*) vaccine candidates and temperature sensitive (*ts*) vaccine candidates developed in the 1970's found these strains were unacceptable for the infant population due to inappropriate attenuation or instability of the *ts* phenotype (Kim et al., 1973, 1971; Wright et al., 1982). Cold passaged temperature sensitive (*cpts*) virus strains, biologically derived by chemical mutagenesis from the original *cp*-RSV vaccine candidate, were shown to be more attenuated than *cp*-RSV, but remained insufficiently attenuated for very young children (Karron et al., 1997; Wright et al., 2000). One of these *cpts* vaccine candidates, *cpts*248/404, was chosen for further development because of its favorable genetic stability and attenuation in RSV naive infants (Belshe et al., 2004; Wright et al., 2000).

Two recombinant RSV vaccine candidates, derived by the introduction of additional attenuating markers into the *cpts*248/404 genomic backbone, have recently been evaluated in infants (Karron et al., 2005). The first candi-

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date, rA2cp248/404ΔSH, is a recombinant analogue of *cpts*248/404 from which the non-essential gene encoding the SH protein has been deleted (ΔSH). Although deletion of the SH gene attenuates wild type RSV A2 in chimpanzees (Whitehead et al., 1999a), rA2cp248/404ΔSH was found to be essentially identical to *cpts*248/404 in its level of attenuation, immunogenicity, and genetic stability in seronegative children 6–24 months of age (Karron et al., 2005). The second recombinant candidate, rA2cp248/404/1030ΔSH, incorporates an additional *ts* mutation, 1030ts, into the genomic background of the first recombinant strain. This mutation, corresponding to amino acid 1321 in the L protein, was identified in one of the original *cpts* vaccine candidates and has been shown to significantly augment the *ts* phenotype of *cpts*248/404 (Crowe et al., 1994; Whitehead et al., 1999b). In contrast to *cpts*248/404 and rA2cp248/404ΔSH, the rA2cp248/404/1030ΔSH vaccine candidate appears to be suitably attenuated for young infants. Notably, however, virus isolates that had partially reverted in *ts* phenotype were recovered from 7 of 53 (13%) RSV-naïve vaccine recipients.

To optimize the design and preclinical evaluation of potential candidate vaccine strains, we sought to better understand why introduction of the 1030ts mutation appeared to reduce the genetic stability of rA2cp248/404/1030ΔSH. RNA viruses have relatively high mutation rates due to lack of a proofreading mechanism in the viral RNA-dependent RNA polymerase (Moya et al., 2000; Smith and Inglis, 1987); however, the rate at which a specific genetic alteration appears in a population is influenced by genetic context and selective pressures, and may vary significantly as these parameters change. All three *ts* vaccine candidates, *cpts*248/404, rA2cp248/404ΔSH, and rA2cp248/404/1030ΔSH, are fully permissive for virus growth at 32 °C and are genetically stable when passaged in vitro at temperatures ranging from 30 to 32 °C, as occurs during the preparation of virus stocks and manufacturing seeds. These virus strains differ, however, in the lowest temperature that restricts their replication: growth of rA2cp248/404/1030ΔSH is significantly restricted at 35 °C, whereas the replication of *cpts*248/404 and rA2cp248/404ΔSH does not become significantly restricted until the growth temperature reaches 37 °C. The upper temperature of the human airway extends up to 35.5 °C (McFadden et al., 1985); consequently, of the three vaccine candidate strains, only rA2cp248/404/1030ΔSH would encounter restrictive replication temperatures resulting in selective pressure to alter its *ts* phenotype. If virus growth-restrictive temperature is a critical factor contributing to the stability of *ts* vaccine candidates in vaccine recipients, then phenotypic and genetic changes similar to those observed in clinical studies should occur under similar in vitro conditions. Therefore, we selected in vitro parameters to simulate growth conditions encountered in the human respiratory tract; specifically, we serially passaged the vaccine candidate strains in a highly permissive cell line (Vero), at 32 and 35 °C. We then compared the *ts* phenotype and genotype of in vitro passaged virus with that of virus shed during clinical studies. In addition, we evaluated whether modifying the 248ts site to reduce the probability of amino acid reversion affected stability of the *ts* phenotype.

2. Materials and methods

2.1. Cells and viruses

Vero cells (Swanson et al., 1988) between passage 134 and 160, and HEp2 cells (ATCC CCL23) between passage 368 and 400, were grown at 37 °C in Eagle's modified minimum essential medium (MEM[E]) supplemented with 2 mM non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25 mM HEPES buffer (Gibco-BRL, Gaithersburg, MD). Once infected, cells were maintained in medium containing 2% FBS.

The *cpts*248/404 and rA2cp RSV were provided by Drs. Steve Whitehead and Brian Murphy (Crowe et al., 1994; Whitehead et al., 1998). The recombinant strains, rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH, were previously described (Karron et al., 2005).

The full-length clone of rA2cp248s/404/1030ΔSH was generated using PCR site-directed mutagenesis to incorporate the 1030ts mutation and, using a reverse genetics strategy previously described, to delete the SH gene (Elliott et al., 2004). The 248ts codon (amino acid 831 in the L protein) was altered from CTG to TTA, so that two nucleotide changes, rather than a single base change, are required for reversion to the wild type amino acid (glutamine; CAA or CAG). Recombinant virus was rescued as previously described (Elliott et al., 2004). The rA2cp248s/404/1030ΔSH strain is identical to rA2cp248/404/1030ΔSH in amino acid sequence, but lacks the six non-coding restriction sites engineered into the L gene of rA2cp248/404/1030ΔSH (Whitehead et al., 1999b).

All reference and vaccine strain viruses used in this study were biologically cloned by plaque-purification or terminal dilution, and were grown in Vero cells at 32 °C. The expected genomic sequences were confirmed by RT-PCR and cycle sequencing using an ABI 377 automated sequencer as described previously (Elliott et al., 2004).

2.2. Virus titration and characterization of the ts phenotype

RSV was titrated by plaque assay in HEp-2 cells incubated at 32 °C under 5% CO_2, as described previously (Belshe et al., 2004). The *ts* phenotypes were assessed by efficiency of plaque formation in HEp-2 cells incubated for 5 days at 32, 36, 37, and 38 °C. The temperature of each incubator was monitored continuously by a chart recorder and verified daily using a National Institute of Standards and Technology calibrated thermometer—to ensure that the temperature never varied more than ±0.2 °C from the set point.