Vaccine 27 (2009) 5822–5829

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Presence of lysine at aa 335 of the hemagglutinin-neuraminidase protein of mumps virus vaccine strain Urabe AM9 is not a requirement for neurovirulence

Christian J. Sauder[a,*], Cheryl X. Zhang[a], Malen A. Link[a,1], W. Paul Duprex[b], Kathryn M. Carbone[c], Steven A. Rubin[a]

[a] DVP/Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA
[b] School of Biomedical Sciences, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom
[c] National Institutes of Health, National Institute of Dental and Craniofacial Research, Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 29 May 2009
Received in revised form 10 July 2009
Accepted 18 July 2009
Available online 4 August 2009

Keywords:
Mumps virus
Urabe AM9 vaccine
Neurovirulence

ABSTRACT

The recent global resurgence of mumps has drawn attention to the continued need for robust mumps immunization programs. Unfortunately, some vaccines derived from inadequately attenuated vaccine strains of mumps virus have caused meningitis in vaccinees, leading to withdrawal of certain vaccine strains from the market, public resistance to vaccination, or in some cases, cessation of national mumps vaccination programs. The most widely implicated mumps vaccine in cases of postvaccination meningitis is derived from the Urabe AM9 strain, which remains in use in some countries. The Urabe AM9 vaccine virus has been shown to exhibit a considerable degree of nucleotide and amino acid heterogeneity. Some studies have specifically implicated variants containing a lysine residue at amino acid position 335 in the hemagglutinin-neuraminidase (HN) protein with neurotoxicity, whereas a glutamic acid residue at this position was associated with attenuation. To test this hypothesis we generated two modified Urabe AM9 cDNA clones coding either for a lysine or a glutamic acid at position 335 in the HN gene. The two viruses were rescued by reverse genetics and characterized in vitro and in vivo. Both viruses exhibited similar growth kinetics in neuronal and non-neuronal cell lines and were of similar neurotoxicity when tested in rats, suggesting that amino acid 335 is not a crucial determinant of Urabe AM9 growth or neurovirulence.
Published by Elsevier Ltd.

1. Introduction

Mumps virus is a member of the genus *rubulavirus* within the family *paramyxoviridae*. Its negative stranded non-segmented RNA genome of 15384 bases encodes nine proteins from seven genes in the order 3′-N, P/V/I, M, F, SH, HN, L-5′. The nucleoprotein (N), phosphoprotein (P), and polymerase protein (L) are responsible for transcription and replication and, along with the genomic RNA, form the ribonucleocapsid. Whereas the P protein is encoded from an alternative open reading frame created by co-transcriptional editing of the P/V gene, unedited transcription of the gene gives rise to the V protein, which is involved in evasion of the host interferon response [1]. Co-transcriptional editing of the P/V gene also gives rise to an additional open reading frame encoding the I protein, although its function has not been established [2,3]. The hemagglutinin-neuraminidase (HN) and the fusion (F) proteins are anchored in the viral envelope and mediate attachment to and fusion with the host cell membrane. Based on inference from other paramyxoviruses as reviewed in [4], the M protein, also membrane associated, plays an essential role in virus assembly and budding. The function of the SH protein is less well characterized, but there is some evidence that it may possess anti-apoptotic activity [5].

Mumps virus exhibits a high tropism for the central nervous system, and, prior to the advent of live attenuated mumps virus vaccines, was a leading cause of encephalitis and aseptic meningitis in developed countries [6]. The incidence of mumps and its complications have decreased dramatically in populations covered by national immunization programs; however, a global resurgence of mumps has occurred over the past few years, highlighting the continued need for robust immunization programs [7–10]. Vaccines derived from inadequately attenuated vaccine strains of mumps virus have caused meningitis in vaccinees, leading to withdrawal of certain vaccine strains from the market or in some cases, cessation of vaccination programs (reviewed in [11]). For example, since the 1993 removal of mumps vaccination from its national immunization program an estimated 1 million cases of mumps occur annually in Japan, causing a rise in mumps induced deafness with an incidence as high as 1/1000 cases [12].

* Corresponding author at: United States Food and Drug Administration, 8800 Rockville Pike, Building 29A, HFM 460, Room 2C20, Bethesda, MD 20892, USA. Tel.: +1 301 827 1977; fax: +1 301 480 5679.
E-mail address: christian.sauder@fda.hhs.gov (C.J. Sauder).
[1] Current address: Schafer Corporation, 3811 Fairfax Dr. Ste #400, Arlington, VA 22203, USA.

0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2009.07.051

Reliably predicting the neurotoxic potential of candidate live attenuated mumps virus vaccines is a challenging task, given that the currently recommended animal model for neurovirulence testing – the monkey model – appears to not be reliable or predictive of virus virulence for humans [13]. Furthermore, despite recent progress [14–17] the molecular basis of mumps virus neurovirulence is unknown and universal genetic markers for mumps virus attenuation or neurovirulence have thus far not yet been identified.

In the case of the Urabe AM9 vaccine, a vaccine strain widely associated with aseptic meningitis and whose use has been largely discontinued, several studies have implicated a single amino acid change with neurotoxicity. The vaccine itself is composed of a mixture of virus variants and those possessing a lysine (K) at aa 335 were reportedly isolated only from vaccinees with postvaccination meningitis or parotitis, whereas variants possessing a glutamic acid (E) at this position were found to be attenuated, suggesting K335 as a marker of neurovirulence and E335 as a marker of attenuation [18–20]. Further supporting this hypothesis, Cusi et al. [21] reported presence of K335 in 10 different mumps virus wild-type isolates. However, this hypothesis was questioned by others reporting that some Urabe AM9 vaccine lots encoding K355 did not lead to adverse events in vaccinees [22,23]. Moreover, K335 also is present in the HN protein of the Jeryl Lynn vaccine strain, a widely used vaccine not causally associated with aseptic meningitis [23]. Finally, in comparing the HN gene sequences of several Urabe vaccine derived isolates, Afzal et al. [19] showed that these sequences differed at several other sites (M89V; N464K; N498D), in addition to aa 335, complicating the interpretation of the initial findings. It later was shown by us and others that the Urabe AM9 vaccine exhibits a high degree of nucleotide heterogeneity across the entire genome [22,24,17,25] making it impossible to determine which genetic change or group of changes is associated with neurotoxicity by simply comparing genomic sequence data.

To overcome this difficulty we generated two cDNA clones of Urabe AM9 that were identical to the consensus sequence of the Urabe AM9 vaccine (Gene Bank no. AF314559) except for changes required to introduce restriction enzyme sites in untranslated regions, 6 silent mutations in coding regions, and four aa changes in coding regions that were chosen based on previous observations of nucleotide heterogeneities at these positions [22,24]. At the amino acid level, the two cDNA clones differed from each other only at position 335 (nucleotide position 7616) in the HN gene, with one virus encoding K335 and the other E335. Both cDNA clones were rescued twice independently. Prompted by a recent report [31] demonstrating a replication advantage of an E335- over a K335-plaque purified variant of Urabe AM9 in a non-neuronal cell line (Vero), yet a replication advantage of the K335 virus in a neuronal cell line (SH-SY5Y), suggestive of increased neurotropism associated with the K335 genotype, we analyzed the replication kinetics of our rescued viruses in these two cell lines as well. Finally, the level of neurotoxicity of the rescued viruses was tested in a rat model for mumps neurovirulence assessment [26,27].

2. Materials and methods

2.1. Cell lines and viruses

Vero (monkey kidney) cells were cultivated in Dulbecco's modified Eagle's medium (D-MEM, Quality Biological (QB), Gaithersburg, MD) supplemented with 9% fetal bovine serum (FBS; QB). BHK-BSR-T7/5 cells [28], kindly provided by Ulla Buchholz (Bethesda, MD), were maintained in D-MEM supplemented with 9% FBS and 1 mg/ml geneticin at every other passage (Invitrogen). SH-SY5Y cells (human neuroblastoma cell line (ATCC no. CL-2266)) were grown in advanced D-MEM/F12 medium (Invitrogen), supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and 9% FBS. The Urabe P-AM9 vaccine virus stock was described before [24].

2.2. RT-PCR, PCR, subcloning and sequencing

Some templates for PCR to generate expression plasmids and Urabe full-length cDNA clones were obtained from archival cDNA fragments derived from Urabe P-AM9 RNA [24]. Other templates for PCR were generated by RT using archival samples of RNA prepared from Vero cells infected with Urabe P-AM9 [24]. All RT reactions were carried out using the superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA).

If not stated otherwise, expand high fidelity Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN) was used for all PCR amplifications. Primers used for generation of expression plasmids and full-length clones are listed in Supplementary Table 1. Subcloning of PCR fragments was carried out using plasmid pCR2.1-TOPO (TOPO TA cloning kit (Invitrogen)). Purified PCR fragments and subcloned PCR fragments were entirely sequenced. Every cloning step was verified by partial sequencing of regions involved in the ligation step. Sequencing was done using an ABI 3100 automated capillary DNA sequencer. Sequence data was analyzed with the Chromas (Technelysium, Tewantin, Australia) and Jellyfish (LabVelocity, Los Angeles, CA) software packages. All obtained sequences were compared to the published sequence of Urabe AM9 "Smith-Kline Beecham live-attenuated vaccine" (Gene Bank no. AF314559; [22]). If not stated otherwise, site directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.3. Construction of plasmids

2.3.1. Expression plasmids

Construction of expression plasmids pMuVNP, pMuVP, pMuVL (kindly provided by D. Clarke, Pearl River, NY), coding for Jeryl Lynn N, P and L genes, respectively has been described elsewhere [29]. Plasmid pTM1mod was derived from plasmid pTM1 (kindly provided by B. Moss [30]) by deletion of a 1238 bp BspEI–NaeI and a 598 bp XbaI–ClaI fragment. Following subcloning and sequencing, the Urabe AM9 virus N, P, and L genes were excised from pCR2.1-TOPO and ligated into pTM1mod as described in Supplementary materials and methods to generate expression plasmids pTM1-NUr, pTM1-PUr, and pTM1-LUr, respectively.

2.3.2. Construction of Urabe full-length cDNA clones $pURABE_{HN\text{-}K335}$ and $pURABE_{HN\text{-}E335}$

A cDNA based clone of the Urabe AM9 mumps virus vaccine strain was assembled using a modified version of the full-length genome cDNA clone pMuVFL [29] that codes for the "JL5" major component of the Jeryl Lynn vaccine. The modified version, termed "pMuV(MPBS)" is identical to pMuVFL except for the inclusion of unique restriction sites (MluI, PmeI, BtrI = BmgBI; SgfI) in the UTRs flanking the M, F, SH and HN genes, respectively [16]. Details of the step-wise assembly of plasmids to produce the full-length Urabe AM9 cDNA clones $pURABE_{HN\text{-}K335}$ (containing a lysine residue at HN amino acid position 335) and $pURABE_{HN\text{-}E335}$ (containing glutamic acid at HN amino acid position 335) is presented in Supplementary materials and methods. The Urabe AM9 cDNAs are flanked by a 5′ T7 RNA polymerase promoter and by a 3′ hepatitis delta virus ribozyme followed by a T7 terminator, resulting in an anti-genome (+) sense RNA transcript following transcription of the cDNA by T7 RNA polymerase (Fig. 1).