The Urabe AM9 Mumps Vaccine Is a Mixture of Viruses Differing at Amino Acid 335 of the Hemagglutinin-Neuraminidase Gene with One Form Associated with Disease

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The reason for the high incidence of vaccine-associated meningitis due to the Urabe AM9 vaccine was assessed by comparing the nucleotide (nt) sequence of the hemagglutinin-neuraminidase (HN) gene from vaccine virus to those of viruses isolated from persons with postvaccination meningitis. A G<sup>1081</sup>→A nt substitution that was predicted to result in a Glu<sup>335</sup>→Lys reversion in the HN protein was detected between Urabe AM9 (G) and postvaccine meningitis mumps virus isolates (A). Further analysis showed that the Urabe AM9 vaccine was a mixture of viruses with wild type (A) and variant (G) nt at position 1081. Urabe AM9 vaccinees who developed meningitis or parotitis possessed predominantly A (98%–100%) at nt 1081, indicating strong selection of the wild type (A) form relative to the variant (G) form. Mumps virus homogeneous for the variant Glu<sup>335</sup> form of the HN gene may be safer than the original Urabe AM9 vaccine.

Mumps virus is a Paramyxovirus of the family Paramyxoviridae, a group of enveloped, nonsegmented, negative-stranded RNA viruses. Infection with mumps virus is established by droplet spread to respiratory mucosa; viremia follows primary replication at this site, leading to infection of many organs. Half of all clinical infections have evidence of central nervous system invasion (as cerebrospinal fluid pleocytosis), usually involving the meninges, and less frequently resulting in meningoencephalitis, which can lead to permanent neurologic damage including deafness [1]. Mumps immunization has been effective at controlling epidemic mumps infection. The measles-mumps-rubella vaccine, Trivirax, produced by Smith Kline & French (SKF; now called SmithKline Beecham) that contained the Urabe AM9 mumps virus strain, was licensed for use in Canada until 1990, when the license for sale was withdrawn due to evidence of adverse effects from vaccination [2]. The rate of meningitis due to Urabe AM9 was ~0.26–3/1000 vaccinations [3].

The reason for the high rate of vaccine-associated disease for Urabe AM9 vaccine is unknown. Sequence analysis of the SH [4] and the matrix and hemagglutinin-neuraminidase (HN) genes [5] of mumps isolates did not identify a role for these genes in the attenuation of Urabe AM9 or for the lack of attenuation associated with mumps isolates obtained after vaccine-induced disease. In this study, we focused our attention on further sequence analysis of the HN gene, since it is a major surface protein of mumps virus and an important candidate gene with respect to vaccine failure. HN protein has two functions, receptor binding, which is required for adsorption of virus to cells (including erythrocytes) and neuraminidase activity, which is thought to promote spread of virus from infected cells [6]. Studies of the pathogenesis of many viral diseases have identified surface proteins as major determinants of virulence [7, 8].

We compared the nucleotide (nt) sequence of the Urabe AM9 HN gene to those of postvaccination clinical isolates associated with disease to determine whether sequence differences could be detected that would explain the manifestation of increased virulence in these specific instances.

Materials and Methods

Cell lines and viruses. African Green monkey Vero cells were obtained from the Bureau of Biologics, Health Canada (Ottawa), and maintained in autoclavable MEM (GIBCO BRL, Burlington, Canada) supplemented with 2 mM L-glutamine and 7% fetal bovine serum (FBS; GIBCO BRL). Urabe AM9 postvaccination mumps isolates from cerebrospinal fluid of 3 patients with meningitis (BB871004 [1004], BB871005 [1005], BB870719 [719]) and throats of 6 patients with parotitis (BB870716 [716], BB870717 [717], BB870718 [718], BB870888 [888], BB870889 [889], BB870890 [890]) and a wild type Canadian isolate (BB870891 [891]) were obtained from the Bureau of Biologics, Health Canada. We obtained two lots of Urabe AM9 vaccine: lot J0547 (URA/PM; Institut Pasteur Mézières, Lyon, France) and lot MP09A44K (URA/SKF; Smith Kline & French, Rixensart, Belgium), which was distributed in Canada before 1989.

Stocks of all viruses were prepared by infecting Vero monolayers at an MOI of 0.05–0.1, then harvesting supernatants at day 5. Stocks were titrated by adsorption to Vero cells in 35-mm plates...
overlaid with 0.6% Noble agar and 0.01% DEAE dextran in 199 medium (GIBCO BRL) supplemented with 7% FBS for 4 days before a second overlay with 0.01% neutral red, which enabled plaque detection on day 5.

Production and cloning of mumps cDNA. Mumps virus was concentrated from cell culture supernatant by polyethylene glycol precipitation. RNA was extracted, reverse transcribed (RT), amplified by polymerase chain reaction (PCR), and cloned as described previously [2]. The entire HN gene was amplified as two or three overlapping pieces using two or three sets of primers in various combinations (mumps sequences taken from Kovamees et al. [9] are bold): 5′-2 (5′-CACGGATCCCAAGTTGAGAATTGGCTCC-3′, HN nt 1783–1803); HNS′+212 (5′-TTATTGTTATGGTCAC-3′, HN nt 212–229); SH+3246 (5′-ATCGGATCCAGCTGGTTCATAGGG-3′), Primer (100 ng) was end-labeled in a 20-μL reaction with 2.5 U of MseI overnight. The proportion of end-labeled mumps virus cDNA produced by RT-PCR was done directly on pools of cDNA produced by RT-PCR.

**Results**

Sequence analysis of mumps virus HN genes. To identify the genetic changes associated with failure of the Urabe AM9 mumps virus vaccine that resulted in meningitis and parotitis, we compared nt sequences of the HN gene of Urabe AM9 vaccine and of 2 Urabe/ASKF isolates (1004 and 1005) from patients who developed meningitis after vaccination. Each HN gene was 1893 nt with an open-reading frame from positions 79-1824 that encoded a 582-aa protein.

Postvaccination meningitis isolates 1004 and 1005 were identical: Both possessed 2 nt differences from Urabe/ASKF, 1 of which produced a coding change from Glu335 (GAA) to Lys335 (AAA) due to a G1081→A substitution relative to the Urabe/ASKF sequence. The published sequence of the HN gene of Miyahara wild type strain from Japan also has a Lys335 residue, suggesting that the Urabe/ASKF vaccine may have acquired the Glu335 mutation upon attenuation.

Urabe AM9 vaccine is a mixture of viruses that differ at nt 1081. Sequence analysis of 4 additional HN cDNA clones derived from the Urabe/ASKF vaccine indicated that 2 clones had the variant codon GAA and 2 had the wild type codon AAA (data not shown). The HN sequence of all of these cDNAs possessed characteristic Urabe AM9 sequences, obviating the possibility of contamination by cDNA clones derived from other mumps viruses in the laboratory (data not shown). It appeared, therefore, that the Urabe/ASKF vaccine was a mixture of viruses that differed in sequence at nt 1081 of the HN gene. Sequencing was done directly on pools of cDNA produced by RT-PCR of viral RNA from vaccine and postvaccine isolates to assess the heterogeneity of sequence at this position. The HN sequence for the Urabe/ASKF vaccine (Vero passage 3) had both an A and a G at position 1081, whereas all postvaccine isolates from patients possessed only an A at this position (figure 1).

The nt change A1081→G seen in Urabe/ASKF results in the loss of an MseI restriction endonuclease cleavage site TTAA to TTGA. This was used for quantifying the relative abundance of the A and G forms in mumps virus stocks by MseI cleavage of end-labeled mumps virus cDNA produced by RT-PCR. Stock Urabe/ASKF (Vero passage 3) was a mixture of 80% G (variant) and 20% A (wild type), whereas all 3 postmeningitis isolates and most (3/5) postparotitis isolates possessed only wild type A at nt 1081. The other 2 parotitis isolates had 2% G and 98% A at this position. This indicates that immunization of individuals with Urabe/ASKF vaccine, a mixture of viruses differing at nt 1081, leads to the selection of strains possessing an A at this position, at least in instances of vaccine-associated disease.

Unpassaged Urabe AM9 vaccines, Urabe/PM and Urabe/ASKF, were mixtures of A and G forms with 54% and 25% variants, respectively. Thus, the Urabe/ASKF vaccine had lower levels of the variant form than the same virus after three passages in Vero cells, indicating a selection of the variant form in Vero cells. To assess the extent of selection of the variant type relative to the wild type in Vero cells, an aliquot of freshly reconstituted vaccine was used to infect Vero cells, followed by serial passage with 1/10th of the culture medium from the preceding passage. The relative amounts of the A and G forms at nt 1081 of the HN sequence after each passage were determined by MseI digestion of cDNA. The relative proportion of the variant G form increased incrementally with passage in Vero cells, from 25% in the vaccine preparation to 68% after four serial passages. Thus, the variant G1081 form had a replicative advantage in Vero cells. This is opposite the selection for the wild type form that occurs in postvaccination disease in humans.

Growth of variant and wild type forms of Urabe AM9. The variant (G1081) and wild type (A1081) forms of mumps virus were plaque-purified from Urabe/PM vaccine to obtain 4 clones of the A-1081 form (A3, A5, A9, A10) and 4 of the G-1081 form (G1, G2, G4, G7). The yield of infectious virus in Vero cells was determined for each of the plaque-purified viruses: Urabe/ASKF vaccine (Vero passage 3; 80% variant form), postvaccination meningitis clinical isolates 1004 and 890, and wild
clinical isolate 891. All of the clinical isolates grew \( \geq 10 \) fold better than the URA/SKF Vero passage three and the plaque-purified variant (G) and wild (A) forms derived from Urabe AM9 (table 1). The A and G forms isolated from vaccine were similar in growth, except for clone 3A. The latter grew less well than the vaccine virus, indicating that some viruses with wild type at nt 1081 are less able to replicate in Vero cells, which is consistent with the selection of virus with the variant sequence in this cell type. This indicates that the growth differences between wild isolates and the vaccine strain were not due solely to the A to G change at nt 1081 and suggests that the clinical isolates of the A1081 form of Urabe AM9 selected in vaccinees who developed clinical disease were genetically different at other genome sites.

### Table 1. Titers of mumps isolates after growth in Vero cells.

<table>
<thead>
<tr>
<th>Mumps virus</th>
<th>Titer ± SD (no. of replicates)</th>
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<tbody>
<tr>
<td>Vaccine and vaccine-like (Glu[^{335}])</td>
<td></td>
</tr>
<tr>
<td>URA/SKF (Vero P3)</td>
<td>( 2.1 \pm 0.8 \times 10^6 ) (8)</td>
</tr>
<tr>
<td>Purified plaques:</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>( 4.4 \pm 2.6 \times 10^6 ) (6)</td>
</tr>
<tr>
<td>G4</td>
<td>( 2.4 \pm 1.3 \times 10^6 ) (6)</td>
</tr>
<tr>
<td>G7</td>
<td>( 2.6 \pm 0.6 \times 10^6 ) (3)</td>
</tr>
<tr>
<td>Wild and wild-like (Lys[^{335}])</td>
<td></td>
</tr>
<tr>
<td>1004</td>
<td>( 3.6 \pm 2.4 \times 10^7 ) (7)</td>
</tr>
<tr>
<td>891</td>
<td>( 4.2 \pm 1.8 \times 10^7 ) (3)</td>
</tr>
<tr>
<td>890</td>
<td>( 3.1 \pm 2.2 \times 10^7 ) (3)</td>
</tr>
<tr>
<td>Purified plaques</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>( 4.9 \pm 0.8 \times 10^7 ) (2)</td>
</tr>
<tr>
<td>A5</td>
<td>( 3.0 \pm 1.9 \times 10^7 ) (5)</td>
</tr>
<tr>
<td>A9</td>
<td>( 5.5 \pm 3.3 \times 10^7 ) (6)</td>
</tr>
</tbody>
</table>

**NOTE.** Vero monolayers were infected at MOI of 0.05 and supernatants were collected and titrated on Vero monolayers at postinfection day 5. Vaccine virus stock was prepared by 3 passages in Vero cells; 1004 is postvaccination meningitis isolate, 890 is postvaccination parotitis isolate, and 891 is wild isolate.

### Discussion

The Urabe AM9 vaccine is a mixture of mumps viruses that differ at a single codon of the HN gene. The wild type HN sequence has an A residue at nt 1081 and encodes a lysine at aa 335, whereas the variant HN sequence has a G residue at nt 1081 that encodes a glutamic acid. The Jeryl Lynn mumps vaccine also contains a mixed virus population [13]. Vaccination with Urabe AM9 that resulted in disease was associated with the wild type sequence, indicating a strong selection in humans of this virus relative to the variant type. It is not clear from these data that this site is instrumental in controlling attenuation or that the A form is more pathogenic than the G form. The data indicate that the A form has a selective advantage relative to the G form during human infection, at least in infections that cause disease, and thus that the A form is pathogenic. It is not known what selection, if any, occurs after uncomplicated vaccination with Urabe AM9 vaccine. An implication of these data is that a vaccine composed entirely of the variant form would be safer than Urabe AM9 vaccine.

The Lys\[^{335}\]→Glu change leads to a reversal of charge in a conserved region of HN adjacent to a neutralization epitope (between codons 352 and 360) in the Kilham strain of mumps virus, which was identified by mapping neutralizing antibody escape mutants [14]. The monoclonal antibody used for selecting mutants also inhibited hemagglutinin and neuraminidase activities of the Kilham virus, indicating that these functional sites are affected by binding of this region of the HN protein [15].

Replication of Urabe AM9 vaccine in Vero cells favors replication of the variant over the wild type form, suggesting that at least a subset of the G1081 variant form has a selective growth advantage. Vero cells may be a preferred host for replication of plaque-purified preparations of the G1081 variant of Urabe AM9 virus. Our results indicate that differences in growth in Vero cells between wild strains and Urabe AM9 are not due solely to the change seen at nt 1081. Further molecular
analysis of cDNAs from the Urabe AM9 virus mixture will address this issue. In the future, we also will assess the safety and efficacy of the purified variant form of Urabe AM9 as a candidate for an improved live attenuated mumps virus vaccine.

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References