CONCISE COMMUNICATIONS

The Mumps Virus Neurovirulence Safety Test in Rhesus Monkeys: A Comparison of Mumps Virus Strains

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Wild type mumps viruses are highly neurotropic and a frequent cause of aseptic meningitis in unvaccinated humans. To test whether attenuated mumps viruses used in the manufacture of mumps vaccines have neurovirulent properties, a monkey neurovirulence safety test (MNVT) is performed. However, results with several mumps virus MNVTs have raised questions as to whether the test can reliably discriminate neurovirulent from nonneurovirulent mumps virus strains. Here, various mumps virus strains representing a wide range of neuropathogenicity were tested in a standardized MNVT. A trend of higher neurovirulence scores was observed in monkeys inoculated with wild type mumps virus versus vaccine strains, although differences were not statistically significant. Results indicated the need for further examination and refinement of the MNVT or for development of alternative MNVTs.

Mumps virus, a member of the Paramyxoviridae family, is a common childhood pathogen possessing highly neurotropic properties. Central nervous system (CNS) infection occurs in about half of all mumps cases, and aseptic meningitis is the most frequent CNS clinical outcome [1, 2].

In this era of high vaccination coverage, some cases of aseptic meningitis have been attributed to vaccination with relatively neurovirulent mumps virus vaccine strains (e.g., Urabe AM9 and Leningrad-3) [3–6]. Although the neurologic safety of mumps virus vaccine seed strains is assessed in monkeys, published reports of clinical and pathologic outcomes of such testing have not always correlated with the virus strain’s neurovirulent potential in humans [7–9]. Multiple factors may contribute to this discrepancy. First, the mumps virus monkey neurovirulence test (MNVT) was modeled after the poliovirus MNVT and does not involve examination of mumps virus-specific targets, such as the brain ventricular system. Second, testing methods for mumps viruses are not standardized. Many scientists use differing numbers of virus doses, routes of inoculation, and scoring systems, resulting in difficulties in interpretation and a lack of uniform criteria for passing or failing a specific virus preparation. Finally, it is possible that the monkey is an inadequate animal model for mumps virus human neurovirulence testing. To address these concerns, we modified a previously published MVNT protocol [10] and performed a standardized mumps virus-specific MNVT, using several mumps virus strains representing a range of CNS pathogenicity.

Materials and Methods

Mumps virus strains. One of the following 7 mumps virus strains was inoculated into each group of monkeys: (1) Jeryl Lynn (JL), the mumps virus component of MMR II trivalent vaccine (Merck, Sharpe & Dohme, West Point, PA) [7]; (2) RIT-4385 (RIT), the JL-derived mumps virus component of Priorix trivalent vaccine (SmithKline Beecham Biologicals, Rixensart, Belgium) [11]; (3) Kilham, a rodent, neuroadapted wild type mumps virus strain (Jerry Wolinsky, University of Texas, Houston); (4) LO1, wild type mumps virus strain isolated from a throat swab of a patient with mumps parotitis (Phil Minor, National Institute for Biological Standards and Control, Hertfordshire, UK); (5) 87-1004, a Urabe AM9 vaccine strain isolated from the cerebral spinal fluid (CSF) of a patient presenting with meningitis post-Urabe AM9 vaccination (Bureau of Infectious Diseases, Health Canada, Ottawa); and (6) AW15 and (7) GW7, two plaque-purified clones of the Urabe AM9 vaccine, differing at position 1081 (A=G, respectively) of the hemagglutinin-neuraminidase gene, prepared, as described elsewhere [5]. Urabe virus strains with A1081 have been associated with aseptic meningitis in vaccinees, whereas virus strains with G1081 have not [6].

Inoculation of monkeys. We used 44 mumps virus–seronegative juvenile rhesus Macaca mulatta monkeys from a domestic breeding colony. Monkeys were anesthetized and inoculated with 0.5 mL of JL (n = 12), RIT (n = 12), Kilham (n = 3), LO1 (n = 3), 87–1004 (n = 3), GW7 (n = 3), or AW15 (n = 3) into each thalamus, as described elsewhere [10]. All virus preparations contained 4.5 log10...
pfu of virus per milliliter. As negative controls, monkeys were inoculated with an equivalent volume of diluent (n = 5).

Monkeys were observed daily for 17 days for clinical signs of disease (e.g., weakness, malaise, parotitis, or orchitis). At the end of the observation period, monkeys were anesthetized and killed by exsanguination. Whole blood, serum, CSF, and a 1-cm³ sample of brain cortex were collected prior to perfusion with PBS. The brain was then removed and fixed in 10% formalin.

Serology. Mumps virus neutralizing antibody titers were determined using a plaque reduction assay against wild type strain LO1 and quantified according to the Kärber method [12]. Seroconversion was defined as neutralizing antibody titer ≥1:8, based on the limits of assay sensitivity.

Infectivity assays. The presence of mumps virus in monkey peripheral blood mononuclear cells (PBMC), CSF, and brain samples was assessed by cocultivation and reverse transcription–polymerase chain reaction (RT-PCR). Monkey tissues and fluids were cocultured on Vero cell monolayers and observed daily for 2 weeks for cytopathic evidence of mumps virus infection, as described elsewhere [13]. RNA for RT-PCR analysis was extracted either directly from PBMC, CSF, and brain samples or after inoculation of tissue culture cells, as described above. Extracted RNA was reverse transcribed into cDNA using primer P1 (5'-TCAGAA-GAATCAACTTTCAAAAACAAAT), corresponding to nt 6088–6113 of the genome. cDNA flanking and including the small hydrophobic gene was amplified by PCR using primers P1 and P2 (5'-GCCGGAACACAGTTGTGATAGCAG), corresponding to nt 6579–6802 of the genome. PCR products were visualized on 1% agarose gels containing ethidium bromide.

Histologic evaluation. Each formalin-fixed brain was sectioned, as previously described, to expose the ventricular system, the target area for mumps virus encephalitis [9, 10]. Brain sections were embedded in paraffin and stained with gallocyanin.

Lesion evaluation. All histologic examinations were done in a blinded fashion. The severity of the histologic lesions was graded on a 0 to 4+ scale as follows: grade 0, no virus-related lesions; grade 1, a combination of 1 or 2 small foci of subependymal infiltrates or perivascular cuffs, inflammation in the choroid plexus or loss, degeneration, or proliferation of ependymal cells; grade 2, a combination of 3–5 small-to-moderately–sized individual foci of lesions as described for grade 1; grade 3, a combination of ≥6 focally extensive lesions as described for grade 1, involving up to half of the ventricle; grade 4, overwhelming severe inflammation and ependymal changes, involving the majority of the ventricle. Representative histologic sections are shown in figure 1. A group histologic lesion score (GHLS) representing each group of monkeys was calculated. Statistical analyses of lesion scores were performed by a one-way analysis of variance with inoculum as the dependent variable.

Results

Clinical findings. None of the monkeys showed signs of clinical disease during the 17-day observation period.

Serology. Neutralizing antibody (≥1:8) to wild type mumps virus was detected in some monkeys from each virus-infected group, but in none of the diluent-inoculated negative control group (table 1). Neutralizing antibody titers ranged from 1:8 to 1:38.

Infectivity assays. No cytopathic evidence of infectious mumps virus was detected after coculturing monkey PBMC, CSF, or brain on Vero cell monolayers. Mumps virus genome was not detected by RT-PCR in samples from any monkeys, assayed either directly or after cocultivation on Vero cells.

Histologic findings. Successful inoculation of all monkeys was confirmed by the presence of either needle tracts in the thalamus or virus-specific inflammatory lesions of the ventricular regions. The average neuroanatomic lesion scores and the GHLSs are shown in table 1. Although most lesions were minimal to moderate, severe lesions were observed in histologic sections from some monkeys in all virus-inoculated groups.

On the basis of the GHLSs, the following comparisons were made: (1) JL to RIT, two related vaccines, (2) JL to wild type strains LO1 and Kilham, and (3) among the 3 Urabe AM9 vaccine strain variants. In the first comparison, the GHLSs of the JL- (1.46 ± 0.26, n = 12) and RIT- (1.46 ± 0.23, n = 12) inoculated monkeys were nearly identical (F(1,23) = 0.00002, P = .996). In the second and third comparisons, although statistical analysis was limited by the low numbers of monkeys per group, there was a trend of increased GHLSs of the wild type strains LO1 (1.76 ± 0.15, n = 3) and Kilham (1.69 ± 0.20, n = 3) relative to JL (1.46 ± 0.26, n = 12) and of increased GHLSs of Urabe AM9 variants AW15 (1.49 ± 0.54, n = 3) and 87–1004 (1.20 ± 0.24, n = 3) relative to GW7 (1.13 ± 0.19, n = 3).

Discussion

To date, published MNVT results have shown an inconsistent relationship between the neuroattenuation state of mumps viruses for humans and the resultant neurovirulence activity in monkeys [7–9, 14]. The variable nature of these MNVT results may, in part, be due to the fundamental lack of test standardization. In all of the aforementioned tests, routes of inoculation were diverse, virus dose and use of appropriate controls were variable, and methods for neuroanatomic evaluation were dissimilar.

To better assess the relevance of monkeys in predicting the neurologic safety of mumps viruses for use in humans, we used a standardized MNVT adapted from a previously published procedure [10]. Here, M. muculatta monkeys were inoculated intrahalamically with ~1 human vaccine dose of several different mumps virus strains representing a wide range of pathogenicity. Each group of monkeys was assigned a neurovirulence score (GHLS), which was based on a blinded, biologically meaningful pathologic evaluation of the ventricular and periventricular areas of the brain, as described elsewhere [9, 10].

Clinical disease was not observed in any of the monkeys nor was the virus recovered by culture or RT-PCR from any of the samples examined. Furthermore, only a minority of the mon-
Figure 1. Galloacyanin-stained coronal sections through lateral ventricles of mumps virus-inoculated monkeys representative of grade 0-4 lesions, A–E, respectively. Areas of choroiditis (within boxes) and periventricular encephalitis (arrows) are indicated. Bar = 125 \( \mu \)m.
keys seroconverted, and the levels of neutralizing antibody were low. Such findings were not due to a lack of infection, since all but 1/39 virus-inoculated monkeys developed mumps virus–specific CNS lesions.

In comparing the 2 related mumps virus vaccines, we found no statistically significant neuropathologic differences between monkeys inoculated with JL and RIT. The statistical analysis of lesion scores supports a hypothesis of equivalent neurovirulence test results, a finding that is not unexpected, since RIT was derived from JL [11]. Of interest, both wild type mumps virus strains (LO1 and Kilham) resulted in greater GHLSs compared with JL. Although the MNVT appeared to correctly discriminate the 2 vaccine strains from the 2 wild type strains, differences were small and statistical analysis was limited, perhaps owing to the few monkeys in the wild type–inoculated groups. Similarly, the GHLSs associated with the 3 variants of the Urabe AM9 vaccine strain showed a trend in order of presumed neurovirulence potential for the human CNS: AW15 (possessing the wild type nucleotide A1081) yielded the highest GHLS, followed by 87–1004 (postvaccination meningitis isolate from human CSF) and GW7 (possessing the potentially attenuating genotype) in order of decreasing GHLSs. Again, because of the few monkeys per group, statistical analysis of the differences in GHLS was limited. It is important to note that the GHLSs of monkeys inoculated with the 3 Urabe AM9 vaccine–derived variants were lower than the GHLSs calculated for monkeys inoculated with JL. This outcome is unexpected, given that vaccination with Urabe, but not with JL, has been causally associated with neurologic complications in humans [4].

There are probably several explanations for the overall inability of the MNVT to conclusively discriminate neurovirulence among virus strains in this study. Given the small differences in GHLSs, in contrast to the wide range in human neurovirulence potential of the virus strains tested, a valid MNVT may require larger groups, a prohibitive feature of the MNVT, considering the logistics and expense of primate testing. Another consideration is that the dose used in this experiment may not have been optimal for detection of virus strain–specific differences in neuropathology. Thus, investigating a range of inocula potencies in the MNVT may be necessary. Ventricular and periventricular inflammation were the primary neurologic end points measured in this test. Although these end points are biologically meaningful, investigation of other parameters, such as the degree of neuroinvasiveness of the various virus strains, may prove to be more discriminatory [14].

In light of the complications and inconsistencies surrounding the MNVT, alternative approaches should be developed (e.g., a nonprimate model of mumps virus neurovirulence) or, as for poliovirus, identification of genomic markers of neurovirulence [15]). We recently described a rat model that can discriminate a wild type from a vaccine strain of mumps virus [13]. Preliminary data from the rat model with virus strains used in this experiment showed discrimination of wild types from attenuated virus strains (unpublished data), suggesting that alternative species may be preferable for neurovirulence safety testing.

Acknowledgments

We thank Mikhail Pletnikov for technical assistance and R. Lundquist, K. Chumakov, P. Minor, and M. Afzal for helpful discussions.

Table 1. Percentage of seropositive monkeys (% SP) and average neuroanatomic lesion scores of anterior lateral ventricle (ALV), inferior lateral ventricle (ILV), posterior lateral ventricle (PLV), third ventricle (3V), fourth ventricle (4V), and Sylvius aqueduct (SA), calculated for each group of monkeys.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No.</th>
<th>% SP</th>
<th>ALV</th>
<th>ILV</th>
<th>PLV</th>
<th>3V</th>
<th>4V</th>
<th>SA</th>
<th>GHLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>JL</td>
<td>12</td>
<td>33</td>
<td>1.85 (0.35)</td>
<td>0.87 (0.23)</td>
<td>1.31 (0.36)</td>
<td>1.71 (0.28)</td>
<td>1.62 (0.30)</td>
<td>1.67 (0.33)</td>
<td>1.09 (0.34)</td>
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<tr>
<td>JL-RIT</td>
<td>12</td>
<td>50</td>
<td>1.87 (0.27)</td>
<td>1.80 (0.87)</td>
<td>1.50 (0.33)</td>
<td>1.88 (0.31)</td>
<td>1.28 (0.26)</td>
<td>1.60 (0.24)</td>
<td>1.27 (0.33)</td>
</tr>
<tr>
<td>LO1</td>
<td>3</td>
<td>33</td>
<td>2.08 (0.08)</td>
<td>1.92 (0.46)</td>
<td>1.50 (0.50)</td>
<td>1.92 (0.33)</td>
<td>1.00 (0.29)</td>
<td>2.28 (0.31)</td>
<td>1.50 (0.50)</td>
</tr>
<tr>
<td>Kilham</td>
<td>3</td>
<td>33</td>
<td>2.08 (0.30)</td>
<td>1.11 (0.49)</td>
<td>2.00 (0.58)</td>
<td>1.78 (0.24)</td>
<td>1.00 (0.29)</td>
<td>2.17 (0.17)</td>
<td>1.67 (0.33)</td>
</tr>
<tr>
<td>87-1004</td>
<td>3</td>
<td>67</td>
<td>1.25 (0.25)</td>
<td>1.17 (0.22)</td>
<td>1.83 (0.60)</td>
<td>1.83 (0.33)</td>
<td>0.83 (0.60)</td>
<td>0.83 (0.33)</td>
<td>0.67 (0.33)</td>
</tr>
<tr>
<td>AW15</td>
<td>3</td>
<td>33</td>
<td>1.25 (0.38)</td>
<td>1.33 (0.33)</td>
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<td>1.42 (0.55)</td>
<td>1.00 (0.58)</td>
<td>1.83 (0.17)</td>
<td>0.67 (0.67)</td>
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<tr>
<td>GW7</td>
<td>3</td>
<td>100</td>
<td>1.06 (0.19)</td>
<td>1.64 (0.49)</td>
<td>1.83 (0.93)</td>
<td>1.22 (0.22)</td>
<td>1.00 (0.29)</td>
<td>1.17 (0.33)</td>
<td>0.33 (0.33)</td>
</tr>
<tr>
<td>Vehicle</td>
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<td>0</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
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</table>

NOTE. Data in parentheses are mean SEs. Seropositive monkeys were defined as having neutralizing anti–mumps virus antibody titer ≥1:

8. Group histologic lesion score (GHLS) represents mean of mean lesion scores for all monkeys/inoculated group.

References

8. Yuzepchuk SA, Rozina EE, Kaptsova TI, Kulish EA. Morphological dif-


