A Single Amino Acid Substitution in the Murine Norovirus Capsid Protein Is Sufficient for Attenuation In Vivo

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MURINE NOROVIRUS (MNV), a prevalent pathogen of laboratory mice, shares many characteristics with human noroviruses. Previous results indicated that passage of MNV1 in the macrophage cell line RAW 264.7 results in attenuation in STAT1-deficient mice (C. E. Wobus, S. M. Karst, L. B. Thackray, K. O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green, and H. W. Virgin, PLoS. Biol. 2:e432, 2004). Sequence analysis revealed two amino acid differences between the virulent and attenuated viruses. Using an infectious cDNA clone of the attenuated virus, we demonstrated that a glutamate-to-lysine substitution at position 296 in the capsid protein (VP1) is sufficient to restore virulence in vivo, identifying, for the first time, a virus-encoded molecular determinant of norovirus virulence.

The human noroviruses, positive-stranded RNA viruses of the Caliciviridae family, are a significant cause of viral gastroenteritis. Unlike the human noroviruses, murine norovirus (MNV) efficiently replicates in tissue culture (8, 18). The ability to grow in tissue culture, combined with the well-characterized and inexpensive mouse model, makes MNV an excellent tool for dissecting aspects of norovirus biology (19). In addition, MNV is a significant pathogen in its own right, with many research colonies of laboratory mice testing positive for MNV-specific antibodies (6, 11).

During the development of the tissue culture system, MNV-1 was plaque purified three times in the murine macrophage cell line RAW 264.7 (18). One of the plaque-purified clones (MNV-1.CW1.P1) was lethal to STAT1−/− mice (18). However, a derivative of this virus (MNV-1.CW1.P3) which had been passed two additional times in RAW 264.7 cells was attenuated in vivo (14, 18). Sequencing of the parental and derivative viruses (MNV-1.CW1.P1 and MNV-1.CW1.P3) identified two amino acid differences: a valine-to-isoleucine change in the nonstructural protein NS4 (G2151A) and a lysine-to-glutamate change in the VP1 capsid protein (A5941G) (Fig. 1A). The contribution of each of these mutations to the attenuated phenotype has not been reported to date.

Recent studies have led to the development of two reverse genetics systems, both based on the attenuated MNV-1.CW1.P3 virus, which allow the recovery of a genetically defined norovirus in tissue culture (2, 17). By use of the reverse genetics system based on recombinant fowlpox expressing T7 RNA polymerase and the attenuated MNV-1.CW1.P3 clone as a backbone (pT7:MNV 3′ Rz, as described in reference 2), the mutations A2151G and G5941A were introduced either individually or together and four recombinant viruses were generated: the attenuated parental virus CW1.P3, P1-NS4 (containing only the A2151G change to the CW1.P1 sequence), P1-VP1 (containing only the G5941A change to the CW1.P1 sequence), and CW1.P1 (containing both the A2151G and G5941A changes). The rescued viruses were subsequently passed once in RAW 264.7 cells to generate high-titer stocks and the sequences of the mutated regions were confirmed.

To assess whether the selection pressure previously identified for CW1.P1 would occur using viruses derived entirely from cDNA, three recombinant viruses (P1-NS4, P1-VP1, and CW1.P1) were passaged three additional times in RAW cells. CW1.P3 was not included, as the sequence of this virus was stable even after multiple passages (data not shown). Growth in RAW 264.7 cells resulted in selection pressure at positions 2151 and 5941 (Fig. 1B and C). The passage 1 viruses appear to be unaltered; however, by passage 4 there is wide-scale reversion to the CW1.P3 genotype. This pressure is more prevalent at position 5941 (VP1) than at 2151 (NS4), as is evident by the mixed population present at position 2151 after four passages in tissue culture. These observations also indicate that the selective pressure at each position arises independently, i.e., sequence changes at position 2151 do not arise as a result of the changes at position 5941 or vice versa.

Sequence-verified passage 1 stocks of the four recombinant viruses were used in single- and multistep growth curve experiments (Fig. 2). The single-step growth curve (Fig. 2A) showed insignificant variation in terms of final titers obtained from the four viruses; however, the kinetics of the exponential phase of virus replication did significantly vary. By use of the statistical method detailed in reference 16 (analysis of variation [ANOVA] excluding 0- and 48-h time points), the variation observed between CW1.P1 and CW1.P3 during single-step growth was shown to be statistically significant (P value of <0.05 calculated using the Kruskal-Wallis and Dunn multiple-comparisons tests). This specific analysis of the viral growth phase by ANOVA (Fig. 2A, highlighted region) demonstrated

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that the amino acid changes in NS4 and VP1 confer a stimulatory role to the replication of MNV-1 in RAW cells.

The effect of the mutations was more marked in the multistep growth curves (Fig. 2B). One-way ANOVA (excluding 0- and 72-h time points) again highlighted a statistically significant variation between the CW1.P1 and CW1.P3 viruses ($P$ value of $<0.05$). Interestingly, the final titer of CW1.P1 never exceeded $10^5$ 50% tissue culture infective doses (TCID$_{50}$/ml, almost 100-fold lower than that seen for P1-VP1, indicating that the change in NS4 has an effect on the in vitro growth characteristics of MNV-1 but only in presence of the K/E change at position 296 in VP1, since CW1.P3 and P1-NS4 do not differ.

To assess the virulence of the four recombinant viruses, 7- to 8-week-old 129/Sv STAT1$^{-/-}$ mice (10) bred and housed at Washington University were orally inoculated with $3 \times 10^4$ PFU of sequence-verified passage 1 virus. Within 7 days, all of the mice infected with the P1-VP1 virus and 90% of the mice infected with the CW1.P1 virus had died (Fig. 3). The recombinant CW1.P3 and P1-NS4 viruses did not cause mortality during this 30-day period. These virulence data clearly indicated that the lysine-to-glutamate substitution at amino acid position 296 in VP1 (nucleotide position 5941) is responsible for the attenuation resulting from serial passage in RAW cells.

The VP1 protein is comprised of a shell (S) and a protruding (P) domain. The P domain is further subdivided into P1 (a constant region) and P2, a hypervariable region within P1 (1, 5). Based on structural studies of Norwalk virus (5), the K/E substitution in VP1 occurs in the variable P2 region of the MNV capsid. The P2 domain of human noroviruses contains both immune and cellular recognition sites responsible for receptor binding (13). The mutation in the MNV VP1 which occurs as a result of passage in tissue culture may have affected receptor binding, affinity, or specificity. Although previous reports hypothesized that the lysine-to-glutamate change at amino acid position 296 in VP1 was responsible for the observed attenuation (14, 18), until now, the substitution at amino acid 11 in NS4 (nucleotide position 2151) could not be excluded from playing an ancillary role.

Recently a cryo-electron microscopic reconstruction of intact MNV virions has been elucidated (9). Density mapping showed a great deal of variation between the MNV virion and other calciviruses (including Norwalk virus). The most interesting variation was in the relative elevation of the P domain in

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**FIG. 1.** (A) Schematic representation of the MNV genome. The positions of the two amino acid substitutions identified in MNV.CW1 P1 and MNV.CW1 P3 which arise as a result of passage in tissue culture are highlighted. nt, nucleotide. (B and C) Selection pressure. The three recombinant viruses (P1-NS4, P1-VP1, and MNV.CW1.P1) generated in this study were passaged four times (P1 to P4 as indicated) at a high multiplicity of infection (MOI) (>2) in the murine macrophage RAW 264.7 cell line. At each passage, the sequences at genome positions 2151 and 5941 (indicated by arrows) were determined by reverse transcriptase PCR. Note that the sequence of CW1.P3 was not included, as this did not change at either position over multiple passages in tissue culture.
MNV virions (16 Å above the shell domain). This elevation is much greater than previously seen for other caliciviruses and this may be indicative of a capsid maturation process. However, since a crystal structure of the MNV capsid is currently unavailable, it is difficult to determine exactly the molecular positioning of the K/E substitution. It may be buried, on the exterior face of the capsid (potentially affecting the virus-cellular receptor interaction), or in fact even on the capsid dimerization interface; therefore, hypothesizing any specific mechanism for this attenuation is premature.

In vivo attenuation resulting from a single point mutation in the viral capsid protein is not unprecedented. Single point mutations in the capsids of foot-and-mouth disease virus, infectious bursal disease virus, and adeno-associated viruses have all been demonstrated to have significant effects on both in vitro and in vivo growth characteristics (12, 15, 20). Interestingly, the K/E change in MNV VP1 maps to a region of the porcine enteric calicivirus capsid where mutations previously implicated in attenuation in vivo were identified (3, 4). These observations with porcine enteric calicivirus have yet to be confirmed by reverse genetics but, combined with our findings, appear to point to a possible general mechanism for calicivirus attenuation as a result of tissue culture adaptation.

Significantly, when the sequences of over 45 MNV capsids (not related to the original MNV-1 isolate) were aligned, the majority of viruses had glutamate at position 296 in VP1 (data not shown), as found in the attenuated derivative of MNV-1 (CW1.P3). Three isolates had a glutamine and only two had lysine, as found for the virulent isolate of MNV-1 (CW1.P1). It appears that the glutamate mutation which arose as a result of tissue culture adaptation of MNV-1 CW1.P1 predominates across numerous MNV isolates, suggesting that the lysine residue in MNV-1 is under specific selection pressure in certain genetic backgrounds in an immunocompromised host.

Although MNV-1 infection does not persist with a short period of fecal shedding (7, 8), other MNV strains cause persistent infections, with virus being found in feces weeks after infection (6, 14). The K/E change in VP1 is clearly responsible for the observed attenuation of MNV-1 in the STAT1−/− mouse model and although the mechanism behind this has yet to be elucidated, it is attractive to postulate that this may affect the ability of MNV-1 to cause a persistent or systemic infection.

Interestingly, the substitution in NS4, which is apparently irrelevant for virulence in the STAT1−/− mouse model, does appear to stimulate infection in vitro and may indicate a possible ancillary role in virus replication and/or tissue culture adaptation. Although the function of the NS4 protein has yet to be elucidated, it is likely to play a role in the establishment of viral replication complexes in the infected cell. Adaptive mutations such as those observed may alter the interaction of NS4 with a host cell factor(s) required for virus replication. Similar mutations which might stimulate human norovirus replication in tissue culture may aid the establishment of efficient tissue culture systems for the human noroviruses.

Recent characterization of numerous MNV isolates showed there to be only a single circulating serotype, but despite this and the clear genetic relatedness of the sequenced viruses, the biology and pathogenicities of these strains appeared to vary greatly (14). Future use of reverse genetics systems could provide a powerful tool for dissecting further the molecular determinants of norovirus virulence and the genetic basis of the observed variation in pathogenicity.

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