Reevaluation of the Virulence of Prototypic Strain 15 of Pneumonia Virus of Mice

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Prototypic strain 15 of pneumonia virus of mice (PVM) has been described as being nonpathogenic in mice, in contrast to the mouse-passaged, highly virulent strain J3666. Previous sequence analysis also indicated that strain 15 encodes an attachment G protein that is truncated at the amino terminus, which for the amino terminally anchored protein deletes the cytoplasmic tail. However, we found that PVM strain 15 obtained from the American Type Culture Collection was highly virulent in mice and was essentially indistinguishable on that basis from strain J3666. Sequence analysis showed that this preparation of virus encodes a G protein with an intact cytoplasmic tail; the truncated predicted protein in the previous sequence appeared to be due to a single nucleotide insertion that disrupted the upstream end of the open reading frame and shifted the translational start site to the next downstream AUG. Taken together, the two studies indicate that strain 15 is an inherently virulent strain but that a nonpathogenic variant that was generated during passage in vitro and encodes a truncated G protein exists. Interestingly, the majority sequence of strain J3666 was found to encode a G protein with an extended cytoplasmic tail, suggesting that there is the potential for considerable plasticity in the cytoplasmic tail of the G protein of PVM.

Pneumonia virus of mice (PVM) is a member of the genus Pneumovirus, subfamily Pneumovirinae, and family Paramyxoviridae, together with the more extensively characterized human and bovine respiratory syncytial viruses (RSV). These are enveloped viruses with a genome that is a nonsegmented negative strand of RNA of approximately 15 kb. PVM was first isolated during an incident when the serial passage of lung homogenate supernatants in apparently healthy mice resulted in the development of morbidity and fatal pneumonia (11, 12). However, whether PVM is a virulent pathogen of rodents in nature remains unclear. Reports of naturally occurring infection and disease are rare and limited to immunocompromised animals, and infection of immunocompetent animals may be inapparent or latent (17, 19). PVM can also infect other rodents, and antibodies specific to PVM or a serologically closely related virus have been detected in many rodent species and also in other mammals, including humans (9, 10, 13–15).

PVM strain 15 is one of the isolates originally described by Horsfall and Hahn (11, 12) and is the only strain available at the American Type Culture Collection (ATCC). Recently, PVM strain 15 was described as being nonpathogenic in inbred mice, in contrast to a different strain, strain J3666, that had been continually passaged in mice and results in morbidity and a high frequency of mortality when inoculated intranasally at low dose in BALB/c mice (5–7, 16). Strain 15 is also the PVM strain that has been most extensively characterized on a molecular level, with sequences available for more than half of the genome, excepting only the 3' (leader) and 5' (trailer) ends of the genome and the large polymerase gene L (1–4, 8, 16). The array of genes and the genome map of PVM correspond closely to those of RSV, although there has been considerable nucleotide and amino acid sequence divergence between the two viruses.

Interestingly, previous sequence analysis of individual cloned cDNAs of the G gene of strain 15 indicated that it encodes an attachment G glycoprotein that, by comparison to RSV, lacks the predicted cytoplasmic tail (16). The pneumovirus G protein, exemplified by RSV, is a type II glycoprotein: it is anchored in the membrane by a hydrophobic sequence that is located near the amino terminus, with the carboxyterminal three-fourths of the molecule oriented externally. Furthermore, RSV G is synthesized in two forms resulting from translational initiation at either the first or the second AUG (codons 1 or 48) in the open reading frame (ORF): initiation at the first AUG gives rise to the complete membrane-anchored form, whereas initiation at the second gives rise to an amino-terminally truncated form that is further processed by proteolysis so that all of the cytoplasmic tail and part of the membrane anchor are missing (18). This form is secreted. The published sequence of the G ORF of the pathogenic PVM strain J3666 (16) contains two comparable AUGs (codons 1 and 34: codon 34 of PVM lies next to the junction between the proposed cytoplasmic and transmembrane domains, while codon 48 of RSV G lies ~12 amino acids within the transmembrane domain). The G gene of PVM strain 15 also has two upstream AUGs that align with those of strain J3666 (16) except for a single nucleotide difference in spacing. However, in the published sequence of strain 15 (16), the first AUG is not in the same reading frame as the major G ORF, due to this single nucleotide difference in spacing. Thus, only the second AUG can initiate transcription of the major G ORF and would give rise to an amino-terminally truncated protein that lacks the cytoplasmic tail.

We obtained PVM strain 15 from the ATCC, amplified it by
two passages in BHK-21 cells, and used the cell culture supernatants to infect 6-week-old BALB/c mice by the intranasal route. In the first experiment, mice in groups of 10 were infected with 6 × 10^3, 6 × 10^4, or 6 × 10^5 TCID50 of PVM strain 15 in an 80-µl inoculum per mouse. As a control, five mice received an equal volume of medium instead of the viral inoculum. The mice were closely observed and weighed daily. Unexpectedly, all of the mice that received strain 15 died or were sacrificed in extremis by day 6 (Fig. 1). A second experiment was performed in which mice received 6 × 10^3, 600, or 60 TCID50 of PVM per mouse (these data are combined in Fig. 1 with the data from experiment 1). Remarkably, none of the mice survived the infection. However, the onset of symptoms was dependent on the virus dose, starting at day 2 for the highest dose or day 4 for the lowest dose. Disease signs included ruffled fur and reduced activity, ending with labored breathing and lethargy in the final stage. Also, the infected mice experienced weight loss up to the time of death. The onset of weight loss was delayed for the mice receiving lower doses; thereafter the kinetics of weight loss was similar, but the mice that received the lower doses experienced a longer duration of weight loss. Thus, the virulence of this preparation of strain 15 appeared to be very similar to that described for the pathogenic strain J3666 (5, 6).

To confirm this result, in a third experiment we infected two groups of five mice each with 800 or 8,000 TCID50 of J3666 (the kind gift of Andrew Easton) and observed the same rate and frequency of death as for the comparable doses of strain 15 (data not shown). Therefore, our preparation of strain 15 was highly virulent and comparable to J3666.

We then amplified the G gene of strain 15 by reverse transcription (RT)-PCR from total infected-cell RNA, cloned it molecularly, and sequenced several clones. In parallel, we prepared a consensus sequence by direct analysis of uncloned RT-PCR product. The sequences thus obtained were in agreement with each other and revealed two differences compared to the previously published sequence for the same strain (GenBank accession no. D11129) (16). One of these was a single nucleotide substitution at position 954 of the published G gene sequence (C to G, negative sense), resulting in the substitution of Glu to Gln (amino acid 258 of the published sequence). The second and more significant difference involved the apparent deletion of one nucleotide in a run of U residues (positions 169 to 173; GenBank accession no. D11129). This deletion is illustrated in Fig. 2A, which shows the sequence (negative sense) of nucleotides (nt) 166 to 196 of the strain 15 G gene and the corresponding electropherogram. It shows a run of 4 U residues beginning at position 169, whereas in the published sequence there are 5 residues. This deletion occurred between the first two upstream AUGs in the G mRNA sequence (nt sequence positions 83 and 183, referred to by Randhawa and colleagues [16]) and had the effect of placing them into the same reading frame, such that the G ORF now initiated with the first AUG (position 83) and contained 33 additional codons. This resulted in a strain 15 G protein that contained a predicted cytoplasmic tail of 35 amino acids, identical in length to that predicted for the published sequence of strain J3666 (16) and for RSV. In the electropherogram, there is a small T peak underlying the C peak at position 173 that might indicate a small subpopulation of genomes that contain a fifth U at this position rather than an insertion.

We also determined a consensus sequence for the G gene of strain J3666 from RNA that was obtained postmortem from the lungs of infected mice and sequenced as uncloned RT-PCR product. This sequencing revealed several differences from the previously published J3666 sequence that was determined from individual cloned cDNAs (16). The most striking difference was the substitution of A to U (negative sense) at nt 65, as compared to the previously published sequence for the same strain (GenBank accession no. D11129) (16). One of these was a single nucleotide substitution at position 954 of the published G gene sequence (C to G, negative sense), resulting in the substitution of Glu to Gln (amino acid 258 of the published sequence). The second and more significant difference involved the apparent deletion of one nucleotide in a run of U residues (positions 169 to 173; GenBank accession no. D11129). This deletion is illustrated in Fig. 2A, which shows the sequence (negative sense) of nucleotides (nt) 166 to 196 of the strain 15 G gene and the corresponding electropherogram. It shows a run of 4 U residues beginning at position 169, whereas in the published sequence there are 5 residues. This deletion occurred between the first two upstream AUGs in the G mRNA sequence (nt sequence positions 83 and 183, referred to by Randhawa and colleagues [16]) and had the effect of placing them into the same reading frame, such that the G ORF now initiated with the first AUG (position 83) and contained 33 additional codons. This resulted in a strain 15 G protein that contained a predicted cytoplasmic tail of 35 amino acids, identical in length to that predicted for the published sequence of strain J3666 (16) and for RSV. In the electropherogram, there is a small T peak underlying the C peak at position 173 that might indicate a small subpopulation of genomes that contain a fifth U at this position and thus conform to the published sequence (16). It is tempting to speculate that this group might represent a minority population with an inserted U at this position rather than an insertion.

We also determined a consensus sequence for the G gene of strain J3666 from RNA that was obtained postmortem from the lungs of infected mice and sequenced as uncloned RT-PCR product. This sequencing revealed several differences from the previously published J3666 sequence that was determined from individual cloned cDNAs (16). The most striking difference was the substitution of A to U (negative sense) at nt 65, as shown in Fig. 2B. Randhawa and colleagues (16) had previously noted that the G gene of strain J3666 (but not strain 15) contained a small ORF (nt 29 to 67, potentially encoding 12 amino acids) that preceded the main G ORF and was in the
same reading frame. The substitution at position 65 ablates the stop codon of this small ORF and thus adds an in-frame extension of 18 codons to the G ORF. Interestingly, the consensus sequence also contained a small A peak underlying the T at position 65, raising the possibility that the virus stock contains a subpopulation encoding an A at position 65, the assignment reported by Randhawa et al. (16). The consensus sequence for strain J3666 contained three additional differences relative to the published J3666 sequence, namely substitutions (given in negative sense) at positions 104 (U to C; Ser to Gly), 165 (C to A), and 173 (C to U).
A; Gly to Val), and 1121 (A to U; Ser to Thr). Interestingly, the assignments in the consensus J3666 sequence at positions 65, 165, and 1121 are identical to those of the consensus sequence of strain 15. Compared to the strain 15 consensus sequence, the J3666 G gene consensus sequence is identical in length (1,333 nt) and contains a total of nine nucleotide substitutions. Two of these are associated with changes in amino acid coding assignment, both in the predicted cytoplasmic tail. Also, as noted above, the position-65 change added 18 amino acids to the amino terminus, resulting in a predicted cytoplasmic tail of 53 rather than 35 residues.

We conclude that PVM strain 15 is not inherently a non-pathogenic strain. Indeed, the virus obtained from the ATCC is highly virulent in BALB/c mice and was indistinguishable on that basis from strain J3666. In contrast, the strain 15 pool that was used in previous studies was shown by those researchers to be nonpathogenic in the same mouse strain (5, 7, 16). It likely represents a variant that had become attenuated in cell culture. The idea that strain 15 was attenuated during passage in vitro was also suggested by Randhawa et al. (16); however, the present study shows that attenuation is not inherent to strain 15 but rather is specific to that particular preparation and presumably occurred during its specific passage history. That passage history included BS-C-1 pri mate cells, raising the possibility that the use of nonrodent cells played a role. Our conclusions extend and clarify, rather than contradict, this previous work (16).

The nonpathogenic version of strain 15 was shown to encode a G protein that lacks the cytoplasmic tail (16), whereas the virulent version of strain 15 analyzed in the present study encodes a G protein that contains a complete cytoplasmic tail. In order to determine the genetic basis for the differences in virulence, it will be necessary to determine complete consensus sequences of the genomes of the nonpathogenic and virulent versions of strain 15 as well as for strain J3666. The available incomplete sequence information indicates that these viruses have very few differences in their genomes and encoded proteins (reference 16 and the present study). This result suggests that the truncation in the cytoplasmic tail of G is the basis for the attenuation in the nonpathogenic version of strain 15. It will be important to confirm this by reverse genetic experiments once such a system becomes available for PVM.

Unexpectedly, we found a second type of alteration in the cytoplasmic tail of G, in this case involving strain J3666. Here, the consensus sequence of the G gene showed that it encodes a G protein that is extended at the amino terminus by 18 amino acids compared either with virulent strain 15 or with the published sequence of J3666 (16). This extension was due to a point mutation that, by removing a termination codon, extended the G ORF to an upstream, in-frame AUG (nucleotide position 29). This additional upstream AUG is not present in the strain 15 sequences but could be created in the corresponding position by a single nucleotide substitution. Reverse genetics studies will be necessary to compare the phenotypes of J3666 virus possessing the 18-amino-acid extension, lacking this extension, or lacking the cytoplasmic tail altogether. The present data, taken together with the previous study by Randhawa et al. (16), indicate that there is naturally occurring plasticity in the length of the cytoplasmic tail of G of strains 15 and J3666 that may be a determinant of viral virulence.

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