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Virulence of Influenza A Virus for Mouse Lung

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Abstract. The experimental infection of mouse lung with influenza A virus has proven to be an invaluable model for studying the mechanisms of viral adaptation and virulence. These investigations have identified critical roles for the haemagglutinin (HA) and matrix (M) genes of the virus in determining virulence for mouse lung. For the HA gene, the loss of glycosylation sites from the encoded polypeptide or changes which may affect the pH of HA-mediated endosome fusion have been observed following adaptation. These alterations also have the potential to impact on receptor specificity, β inhibitor sensitivity and activation cleavage which may act in concert to account for the increased virulence of adapted strains. For the M gene, two specific changes in the M_1 protein have been identified in strains adapted to, or virulent for, mouse lung. These changes are likely to affect pH-dependent association/dissociation of M_1 with the viral ribonucleoprotein, and control virulence as well as growth. The role of other genes in mouse lung virulence remains unknown.

Key words: influenza A virus, virulence, mouse lung, HA gene, M gene

Introduction

Mouse Lung Virulence

The ultimate challenge for investigators of viral pathogenesis is to identify the role of each gene in the manifestation of disease. For over sixty years, the experimental infection of mouse lung with influenza virus has provided a tool for the understanding of viral pathogenicity and adaptation (1,2). Initial studies focussed on the basic biology of adapted and unadapted strains, and provided an overall picture of influenza pathogenesis, particularly due to the similarities to human infection (3). Later reassortant studies allowed the identification of the genes involved in mouse lung virulence, or pneumovirulence, which were pivotal in the broader understanding of influenza virulence determinants. However, the more recent advances in molecular virology techniques have allowed the dissection of the genes and genetic changes important in the adaptation process. Our own approach has been to identify sequence changes observed in strains adapted to mouse lung, compared to either parental or non-adapted strains, and attempt to correlate these with biological changes in these isolates. This review aims to meld these different studies together and to present our current understanding of the molecular basis of adaptation to mouse lung.

Infection of Mouse Lung with Non-adapted Strains

Mice are not naturally infected with influenza virus, but most strains can be experimentally infected with the virus. When newly-isolated human influenza A viruses are used to infect mouse-lung, they produce a largely asymptomatic infection of the respiratory tract with no sign of lethal pathogenic effects (4,5). However, the unadapted virus does replicate in lungs, bronchioles and trachea, and is able to reach a high titre (4,5). Virus begins to be cleared from lungs after around 5 days coinciding with an increase in humoral immunity. By the eighth day, considerable regeneration is observed, including the appearance of squamous metaplasia (5). If large inocula of unadapted virus is used, while there is a suppression of viral replication (5,6), mice develop a toxic

pneumonitis and often death, although this is not due to specific adaptation of the virus (6,7).

Adaptation of Influenza to Mouse Lung

Influenza A mutants which induce pathology in the nose, trachea, bronchi or lungs emerge during serial passage of human influenza viruses in mice (4,5,8). Such mutants which can cause lethal pneumonitis have acquired an increased ability to infect alveolar cells, thereby initiating alveolitis (5,8). All epithelial cells of the bronchi and alveoli are susceptible to infection with fully mouse-adapted strains (9,10), with neonatal mice more susceptible to lethal pneumonia than adults (11). Cellular infiltration of the lung, which is a characteristic of lethal infection, is decreased in nude mice indicating a role for T cells in mediating the immune response to influenza infection (8). In addition, the viral pneumonia induced in the mouse shows considerable pathological similarities with the human condition (3). Thus, mouse lung is an eminently suitable model for studies of influenza viral pathogenicity.

Influenza Virulence Determinants

Considerable work has been done in identifying factors which affect influenza host range and virulence. These studies have indicated that alterations in any of the eight genes of the virus can affect virulence or host range, depending on the system under investigation. The surface glycoproteins, neuraminidase (NA) and haemagglutinin (HA), are often the major determinants. For example, the NA is responsible for the replication of A/WSN/33 in MDBK cells (12) and mouse brain (13), of the enterotropism of A/mallard/New York/78 for ducks (14), and is a contributory determinant of mortality of A/chicken/Japan/24 for chickens (15). Similarly, the HA gene has been implicated in increased virulence for avian strains via a basic stretch of amino acids at the cleavage site (16), while amino acid substitutions in the receptor-binding site allow increased replication in ducks (17), swine (18), and the MDBK cell line (19). However, all genes have the potential to impact on virulence, with the matrix (M) gene in particular being a critical determinant of viral replication capacity (20–22).

Study of these virulence determinants have shown that virulence is modular, with specific genes often giving additive effects, as is the case in mouse neurovirulence (23). In other cases, however, a particular set of genes are required *in toto* to achieve virulence—a so-called ''gene constellation'' effect (24). This is probably due to a need for compatibility between individual viral genes, particularly those of the ribonucleoprotein complex, rather than for actual virulence *per se*. For example, four genes of A/Turkey/England/63 virus are required for mouse hepatotropism (25,26).

The Molecular Basis of Mouse Lung Virulence

Genes Responsible for Mouse Lung Virulence

Studies examining the pathogenicity of a number of mouse lung-adapted viruses have indicated that the viral HA is critical in determining virulence for mice (27–32). However, other genes have also been implicated. For example, studies on the mouse lung-adapted A/FM/1/47 have revealed that in addition to the HA gene controlling virulence, the M gene controls virulence and growth (32), while Kaverin et al. (30) have shown the importance of the basic polymerase 1 (PB1) gene in concert with the HA in determining virulence, with a role for the M or nucleoprotein (NP) genes not able to be discounted. In addition, the PB1 gene of A/PR/8/34, and both the acidic polymerase (PA) and PB1 genes of A/England/1/61 have been implicated in mouse lung virulence (26,33), while the PB1 gene appears to be necessary for mouse lung virulence for A/USSR/90/77 in some reassortments (34). The importance of other (unidentified) genes apart from HA has also been shown in the adaptation of A/Aichi/2/68 to mouse lung (34).

Changes in the HA Gene

The HA mediates attachment of host-cell sialyloligosaccharide receptors via a binding site pocket at the distal tip of the molecule, which determines the binding specificity (35–37). In its cleaved form the HA consists of two disulphide linked subunits (HA_1 and HA_2) which are non-covalently associated in an HA trimer (38). Each monomer is 13.4 nm long, consisting of a globular membrane distal region (HA_1 only) on top of an elongated stem (both HA_1 and HA_2) (35). All attached oligosaccharides are N-linked to asparagine and, with the exception of position 165 on

HA_1 (H3 numbering) which extends across an adjacent monomer, all are on the lateral surface of the molecule (39). Cleavage of the HA is essential for infectivity of the virus particle (40,41). This permits fusion of the viral envelope with the secondary endosome, a process activated by conformational changes in the HA at the acidified pH of the endosome which exposes the fusogenic N-terminus of the HA_2 subunit (42,43).

As discussed earlier, the HA is a primary determinant for mouse lung virulence, and a number of studies have examined the changes in the HA associated with increased virulence (Table 1). The most obvious conclusion from these investigations is that the loss of glycosylation sites represents a common theme during mouse lung adaptation. For example, the HA molecule of the mouse lung-adapted A/USSR/90/77 was found to have acquired two successive amino acid substitutions in HA at residue 127 and 89 of HA_1, (corresponding to residues 131 and 96 respectively in H3 numbering). Both of these result in the loss of potential glycosylation sites (at positions 131 and 94a in H3 numbering), with both changes in the vicinity of the receptor-binding site (29,44). Studies with the H3N2 reassortment virus A/Phil/82 has also shown that the successive loss of potential glycosylation sites at residues 165 and 246 is associated with significant stepwise increases in the pathogenicity of this virus for mice (45,46), supporting a role for the removal of carbohydrate in mouse lung-adaptation. Similar loss of carbohydrate attachment sites has been seen in other studies. For example, we have found that the equivalent glycosylation site to that at residue 165 of the H3 strain is lost in the two neurovirulent derivatives NWS and WSN (47). Likewise, the removal of glycosylation sites have been shown to be critical in the virulence of A/Chicken/Pennsylvania/83 (16), and for a mutant of WSN which exhibits increased virulence in an experimental cell model (19). Thus, the loss of glycosylation sites represents a common mechanism for acquisition of virulence.

So, how does the removal of carbohydrate attachment sites contribute to viral pathogenesis in mouse lung? It would seem unlikely to be due to antigenicity, since glycosylation has been shown to mask antigenic sites on the HA (48), and those changes in antigenic specificity observed during mouse lung-adaptation were independent of the increased pathogenicity for mouse (28). However, a number of other mechanisms are possible. For example, loss of carbohydrate from the HA of the WSN strain affected receptor binding specificity (19). This and other changes in receptor specificity have been shown to affect host preference in a number of systems (17,19,49). In support of a role for altered receptor specificity in increasing virulence for mouse lung, we have shown that mouse lung-adapted A/Phil/82/BS/ML10 exhibits both increased mouse lung virulence and altered receptor specificity (46), as do the mouse lung-adapted strains A/NY/43 (4) and A/USSR/90/77-7p (44). Other studies indicate that the removal of a glycosylation site can affect HA cleavage and so regulate virulence (16,50), although no alterations in HA cleavage has been reported in mouse lung-adapted strains. In addition, the loss of carbohydrate sites may be involved in avoiding inhibition by β inhibitors (discussed later). These

Table 1. Changes observed in the HA associated with increased mouse lung virulence

Strain	Change	Comments	Reference
A/USSR/90/77 (H1)	HA_1 $Asn^{127} \rightarrow Asp$	Loss of glycosylation site Resistance to β inhibitor Altered receptor specificity	(29,44)
	HA_1 $Thr^{89} \rightarrow Ala$	Loss of glycosylation site	(29,44)
A/Phil/82 (H3)	HA_1 $Thr^{167} \rightarrow Leu$	Loss of glycosylation site Resistance to β inhibitor	(45,46)
A/Phil/82/BS (H3)	HA_1 $Asn^{246} \rightarrow Ser$	Loss of glycosylation site Resistance to β inhibitor Altered receptor specificity	(46)
A/FM/1/47 (H1)	HA_2 $Try^{47} \rightarrow Gly$	Altered pH of fusion Resistance to β inhibitor	(32)

mechanisms are by no means mutually exclusive and may act in concert to account for the increased virulence following adaptation.

The other change in the HA observed following adaptation to mouse lung is probably involved in HA catalyzed fusion. In this case, the mouse lung-adapted A/FM/1/47 showed a single amino acid change in the HA_2 subunit ($Trp^{47} \rightarrow Gly$) which controls virulence (32). Amino acid 47 of the HA_2 has been shown to change in three amantadine-resistant variants with a concomitant increase in the pH optimum of HA-mediated endosome fusion (51). In addition, we have shown that the mouse lung-adapted strain A/Phil/82/BS/ML10 shows an altered pH of fusion which may allow improved efficiency of replication in mouse lung (46). By analogy, we have identified a change in the mouse neurovirulent strain NWS relative to its parent A/WS/33 at another site shown to affect the pH of fusion (47), while variants of influenza selected for their ability to grow in MDCK cells also have an elevated fusion pH threshold (52). Alterations in the pH of fusion, therefore, may also represent a general pathway for adaptation and increased virulence, which has an important role in adaptation to mouse lung. However, in other systems mutations that affect the pH of fusion can also affect receptor binding (43) or activation cleavage (52), while the mouse lung adapted A/FM/1/47 strain has also become resistant to α and β inhibitors (32). This again highlights the complex interplay of biological factors which must be considered before more precise conclusions can be drawn.

Role of β Inhibitors

β inhibitors of influenza virus were initially described as heat-labile components of normal sera which have virus-neutralizing and haemagglutination-inhibiting activity (53–55). Later studies showed them to be Ca^{2+}-dependent proteins, resistant to neuraminidase and periodate (56), which could inhibit viruses of the H1 and H3 subtypes, but not of the H2 subtype (57). The β inhibitors were eventually identified as mannose-binding proteins which bind carbohydrate at the tip of the HA spike, blocking access of cell-surface receptors to the receptor binding site on HA (58). In addition, these mannose-binding lectins are able to mediate complement-dependent lysis of influenza virus-infected cells, and so form an important arm of the innate defence system of the mouse (59).

Early studies discovered that H1 viruses which had undergone passage in mice were resistant to β inhibitors from bovine and mouse serum (55,60,61), with the acquisition of resistance occurring at the same passage as increased virulence (61). Further investigation of this association showed that mutant viruses that had been selected with serum *in ovo*, while not as pathogenic as fully mouse adapted viruses, were more pathogenic for mice than their β inhibitor-sensitive parent viruses (55,61,62). Thus, it appears β inhibitors may play an important role in the natural immunity of mice to certain subtypes of influenza. Since a number of mutant viruses selected for resistance to β inhibitor have lost a glycosylation site on the HA (45,58), one factor in the development of virulence for mice may be a mutation leading to loss of ability of the mouse inhibitor to bind to the virus, or cells infected with the virus. This would allow the virus to overcome this important component of the innate immunity of the mouse. In support of this, we have shown that the A/Phil/82/BS derivative exhibits loss of a glycosylation site, loss of β inhibitor sensitivity, and acquisition of virulence for mice (46). Interestingly, the adaptation of the A/FM/1/47 virus to pathogenicity for mice was again noted to be accompanied by β inhibitor resistance, even though no glycosylation site was removed, suggesting other structural factors are also important in determining β inhibitor resistance (32).

Changes in the M Gene

The M gene encodes two viral structural proteins, M_1 and M_2, with only the M_1 protein implicated in virulence for mouse lung (32,63). The M_1 protein is the most highly conserved protein of influenza A virus (64). It lines the inner layer of the viral membrane, contacting the ribonucleoprotein (RNP) core (65). The M_1 protein binds to lipid, other M_1 molecules, RNA and RNP, and can regulate gene expression by inhibiting transcription (66). It is released from the RNP during uncoating following acidification of the inner virion (67). The M_1 protein also performs an essential role in promoting export to the cytoplasmic membrane, virus assembly and budding (68–71).

Sequence analysis of parental and progeny strains have allowed the identification of three specific changes which have been observed in the M_1 protein

Table 2. Changes observed in the matrix (M_1) protein following adaptation to mouse

Change	Parent	Adapted progeny	Mouse adaptation	Reference
$Ala^{41} \rightarrow Val$	A/Port Chalmers/1/73	A/Port Chalmers/1/73/-MA	Lung	(72)
	A/WS/33	WSN	Lung and brain	(63)
	A/WS/33	NWS	Lung and brain	(63)
$Thr^{139} \rightarrow Ala$	A/FM/1/47	A/FM/1/47-MA	Lung	(32)
	A/WS/33	NWS	Lung and brain	(63)
$Ala^{227} \rightarrow Thr$	A/WS/33	NWS	Lung and brain	(63)

following adaptation of influenza virus to mouse (Table 2). The $Ala^{41} \rightarrow Val$ change is the *sole* amino acid substitution observed in the mouse lung-adapted derivative of A/Port Chalmers/1/73 (72), and in the pneumo- and neurovirulent strain WSN (63) compared to their respective parents, whilst it is one of three changes in another pneumo- and neurovirulent strain, NWS (63). Multiple sequence analysis of the available M_1 sequences performed in my laboratory (63) has shown that while the Ala^{41} is largely conserved, a Val^{41} is seen in the strain A/PR/8/34, which was extensively passaged in mouse and is also pneumovirulent for mice (24), and in A/FPV/34, where the M gene has been implicated as a neurovirulence determinant for mice (24). The $Thr^{139} \rightarrow Ala$ substitution is the *sole* amino acid change in the M proteins of the mouse lung-adapted variant of A/FM/1/47 compared to its parent (32), as well as the second of the three changes in NWS (63). This change has been shown to increase virulence and growth in mouse lung-adapted A/FM/1/47 (32). All other strains retain a Thr at this position (63). The $Ala^{227} \rightarrow Thr$ is the last of the three changes in the NWS strain. Once again the Ala at this position is highly conserved, with a Thr being only found in only one other strain, A/Equine/Prague/1/56 (63). This highlights the tremendous specificity of those changes responsible for mouse lung virulence.

So, what effects might these changes have on viral replication? The change at position 139 is in the region responsible for RNA/RNP binding (66,73), and it has been suggested that this change may also affect the pH-dependent association/dissociation of M_1 with RNP, thereby controlling virulence and growth (32). It seems likely that the $Ala^{41} \rightarrow Val$ change may also affect growth since the WSN M gene has been implicated in increased growth rate in MDCK cells (22) and *in ovo* (21), while the A/PR/8/34 M gene (which also contains Val^{41}) is likewise a determinant of *in ovo* growth rate (74). In addition, the observed change at position 41 has been correlated with the acquisition of resistance to a monoclonal antibody to the M_2 protein (75). This study implies that such a change in M_1 may compensate for a loss of M_2 function, such as during the passage of the virus through the acidic endosome after endocytosis. In support of the Val^{41} change having a role in this part of the virus life cycle, the WSN M_1 protein has been shown to dissociate at a higher pH than a strain with an Ala^{41} (22). Therefore, both changes are likely to affect pH-dependent association/dissociation and growth, thereby increasing virulence. Smeenk and Brown (32) have argued that the M_1 protein of the adapted variant may bind RNP less strongly, allowing replication to proceed to higher levels. This is an attractive model to be consideration in further studies. The changes in the M_1 observed in mouse lung adaption, seem to represent general determinants of virulence, since they facilitate adaptation to both mouse lung and mouse brain (63), and control virulence in other host cell systems (21,22,74). The role of the mutation at residue 227 (if any) remains unresolved.

Role of the Polymerase Genes

The polymerase genes, PA, PB1 and PB2, encode the components of the influenza virus polymerase complex required for viral transcription (reviewed in 76). The involvement of different members of this complex in the determination of virulence has been reported in a number of systems (27,77,78). As mentioned earlier, both the PA and PB1 genes have been implicated in mouse lung virulence (26,30,33,34,44). However, there is no evidence that the gene has acquired mutations as a result of adaptation to mouse lung. Thus, these particular polymerase genes may simply need to be compatible

with the other viral genes, such as the NP gene, to allow efficient replication to proceed (30), in a manner analogous to neurovirulent recombinants of FPV which require particular polymerase genes from the non-adapted strain (24,70). Further reassortant studies and sequence analysis are required to confirm a positive role for these genes in mouse lung-adaptation.

The Biology of Mouse Lung Virulence

Mechanisms of Mouse Lung-adaptation

Having achieved a comprehensive analysis of the molecular changes responsible for mouse lung virulence, we can now begin to define the mechanistic changes which allow the adapted strain to display its increased virulence and pathogenesis for mouse lung. The enhanced virulence can potentially be a result of a number of factors, including increased viral replication *per se*, or overcoming host restrictions on viral entrance, spread or replication. Raut et al., (5) showed that increased virulence was not due to decreased interferon or antibody production, since these were actually higher in the mouse-adapted strain. However, other aspects of host immunity which limit infection, β inhibitors, altered receptor binding sites, and different cellular environments for mediating uncoating or cleavage activation could still be important.

Consistent features of adapted strains is the removal of a lag phase seen in unadapted strains (5,32,80), attainment of a maximum titre in a shorter time (4,32,46,81), and usually a slightly increased maximum virus yield (5,32,46,82). One possible explanation for this, given the observed changes in the HA and M_1 in adapted strains, is that there is slower viral unpackaging due to reduced kinetics of HA-mediated fusion and M_1-RNP dissociation as a result of a difference in the endosome environment in mouse lung. One could then argue that the adapted strains overcome this impediment by mutations which increase the pH threshold required for these processes, allowing replication to begin more quickly. The M_1 mutant may also bind RNP less strongly, allowing replication to proceed to higher levels. Further studies will be required to confirm this model.

Some authors have argued that increased virulence is not due to increased growth rate, or higher titre, but due to new pathological properties (4) or an ability to induce a more persistent infection (5). In support of this, the mouse adapted variant of the Kunz strain of influenza virus showed a greatly increased ability to infect alveolar cells of the mouse lung to result in rapidly spreading alveolitis and pneumonitis compared to its unadapted parent (5). By analogy, no correlation between growth rate and pathogenicity FPV recombinants for chickens was seen; instead, tropism is the most important factor for expression of virulence (77). However, since increased pathogenicity is consistently correlated with the emergence of an accelerated growth rate, increased virulence is probably a consequence of both increased viral replication and altered tropism. In fact, Smeenk and Brown (32) were able to show that while both HA and M genes determined virulence of mouse lung-adapted A/FM/1/47, only the change in the M gene controlled growth, and concluded that the mutant had an improved ability to replicate. This neatly separates virulence as necessarily involving increased growth rate for the HA gene, while the two may be linked for the M gene. Thus, the M gene would seem to control virulence by enhancing viral replication, while the HA gene contributes to virulence by overcoming host restrictions on viral entrance and spread. Indeed, the surface glycoproteins of other viruses similarly dictate virulence for mouse (83,84). More detailed studies into the biology of these mouse lung-adapted viruses will help identify the exact role of each gene in determining virulence.

Host Factors

While this review focuses on virus-specific factors which determine virulence, host-specific factors are also important. For example, certain strains of mice (for example, the A2G strain) are resistant to neurotropic, hepatotropic, and pneumotropic influenza virus variants (85). Such strains carry a dominant allele (Mx^+) that specifies an influenza virus-specific increase in sensitivity to interferon (76,86). In addition, the presence of specific host major histocompatibility complex haplotype may also be important (87). Clearly, these important factors deserve more detailed consideration if we are to fully grasp the complex interactions between virus and host which determines whether a productive infection occurs.

Conclusions

Mouse lung virulence provides a very useful experimental system for investigating viral adaptation and pathogenesis. The similarities with human infection has permitted useful dissection of the host response to infection, both innate and humoral. Analysis of the genes which determine mouse lung virulence has revealed a great deal about the mechanisms of adaptation. As is the case for mouse neurovirulence (23), the specific changes which result in mouse lung virulence are the consequence of single nucleotide, and resultant amino acid, substitutions. Thus, the inherent high mutation rate of influenza again enables it to overcome restrictions in growth and explore new host cell environments through subtle alterations in its encoded proteins. I would propose to describe this process as ''adaptative drift'', since it so obviously parallels ''antigenic drift'' both mechanistically and in its biological outcome. Studies into the genetics of adaptation have also given further support to the notion that virulence is modular, with genes able to contribute independently to the overall virulence of the virus. Therefore, once a virus has undergone sufficient drift to adapt to a new host milieu, it is then able to increase its virulence further through reassortment with other adapted strains, or ''adaptative shift''.

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