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Review

Neurovirulence of influenza A virus

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Infection of mouse brain with influenza A virus has provided a valuable model for investigating viral adaptation and virulence. These studies have indicated important roles for the neuraminidase (NA), matrix (M), non-structural (NS) and haemagglutinin (HA) genes of the virus in determining neurovirulence. For the NA, three changes close to the active site have been identified in the neurovirulent strains, which also display altered enzyme properties, including changes in specificity. In the M gene, two specific amino acid substitutions in the M_1 protein have been observed, $Ala^{41} \rightarrow Val$ and $Thr^{139} \rightarrow Ala$, which correlate with increased virulence for mouse. Such changes are likely to affect the pH-dependent association/dissociation of M_1 with the viral ribonucleoprotein, as well as growth and virulence. The changes in the NS gene in the neurovirulent strains cause alterations in the mRNA secondary structure to mask the 3′ splice site, and correlate with reduced splicing of the NS gene in these strains. Finally, the increased virulence of the HA gene occurs by at least three different mechanisms: loss of a glycosylation site, a change at the cleavage site, and a substitution which may increase the pH of fusion. These observations define a useful set of parameters with which to analyse epidemic virus strains that have been associated with elevated CNS symptoms in humans. In addition, the changes present in the neurovirulent influenza strains show interesting parallels to those in the neurovirulent derivatives of other viruses, suggesting different viruses utilise common strategies to permit replication in the brain.

Keywords: virus adaptation; neurovirulence; NA gene; M gene; NS gene; splicing control; HA gene; HA cleavage

Introduction

Neurovirulence

Virulence of influenza virus for mouse brain, or neurovirulence, has been an important topic in the development of influenza virus research. Neurovirulence is defined as the ability to undergo multicycle replication in mouse brain, inducing neuropathology and acute encephalitis. In adult mice, this only occurs after intracerebral inoculation, while in newborn mice it can also occur as part of generalised pantropic spread after intranasal infection (Wagner, 1955). Neurovirulence was one of the first markers for recombination and its study has proved pivotal in establishing some of the most important concepts of influenza virulence and pathology. Application of biochemical and molecular biological analysis to this area of research in recent years has unravelled some of the molecular changes required for adaptation to mouse brain, and the molecular mechanisms which mediate neurovirulence. The purpose of this review is to provide an overview of the phenomenon of influenza neurovirulence and to present our current understanding of the process of adaptation of this virus to mouse brain.

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Central nervous system infection

The involvement of the central nervous system during influenza infections in humans is still unresolved, although there appears little doubt that encephalopathy does occur with some patients of influenza (Kilbourne, 1987). For example, a causal relationship has been identified between the 1918/9 pandemic and encephalitis lethargica, with its common manifestation in post-encephalitic Parkinsonism (Ravenholt and Foege, 1982). In addition, the 1957 pandemic produced a number of reports of central nervous system manifestations (Horner, 1958; Sugiura *et al*, 1958). Likewise, the Alaskan influenza epidemic in 1973 provided further evidence for encephalopathy as an atypical manifestation of the disease (Edelen *et al*, 1974). Virus has also been isolated from the brain tissue (Murphy and Hawkes, 1970) and spinal fluid (Thraenhart *et*

al, 1975; Rose and Prabhakar, 1982) of fatal cases of human influenza infection, and viral antigen has been found in ependymal cells (Franková *et al*, 1977). Moreover, during the epizootic in seals resulting from infection with the avian strain A/seal/Mass/80 (H7N7), virus was isolated from both the lungs and brains of dead seals, caused conjunctivitis in laboratory workers, and was capable of replicating in the brains of mice (Webster *et al*, 1981). Therefore, the ability of influenza virus to replicate in the central nervous system of mice has been studied in detail as a model of the central nervous system manifestations of the human disease. While the artificial route of this model means such studies have little relevance to epidemiological aspects of influenza biology, they have provided huge insights into many aspects of influenza virus replication and adaptation.

Typical influenza replication

Influenza A virus particles are surrounded by a host-derived lipid envelope, from which protrude viral glycoprotein spikes, corresponding to the haemagglutinin (HA) trimer and the neuraminidase (NA) tetramer, and which also contains the M_2 protein tetramer (Murphy and Webster, 1990). Beneath this is an electron dense core, composed of matrix (M_1) protein, which interacts with the helical internal ribonucleoprotein complexes (RNPs). These consist of the eight segments of viral genomic RNA (vRNA) surrounded by a nucleoprotein (NP) backbone, with the three polymerase proteins (PB1, PB2 and PA) in close association, attached at their end (Krug *et al*, 1989).

Infection is initiated by the attachment of the viral HA spikes to sialic acid-containing receptors on host cells (Kilbourne, 1987). Once attached, virus-cell fusion occurs via an endocytic pathway, in which the endosomes are acidified by the action of proton pumps (Wharton, 1987). This acidification has two effects: (i) there is a conformational change allowing interaction of the fusogenic HA_2 N-terminus with the cell membrane (Skehel *et al*, 1982), and (ii) the M_1 dissociates from the RNPs (Wharton *et al*, 1989). This allows the RNPs to be released into the cell and enter the nucleus, where replication is facilitated by the four proteins of the RNP (Huang *et al*, 1990). Later in infection the M_1 protein has a role in promoting export to the cytoplasmic membrane by associating with newly synthesised RNPs in the nucleus, and in subsequent virions (Helenuis, 1992). Neuraminidase activity is a prerequisite for elution of virus from infected cells due to its ability to remove receptors from the haemagglutinin by cleaving the terminal sialic acids (Colman, 1989). It probably also prevents virus aggregation, and assists in penetration of the mucous layer in the respiratory tract (Lamb, 1989).

Infection of mouse brain with influenza

Abortive replication in mouse brain

In neonatal mice, several cycles of replication can occur with non-adapted strains (Fraser *et al*, 1959; Mims, 1960). However, in adult mice, the intracerebral administration of non-adapted human virus initiates an abortive infection of ependymal cells, the destruction of which can lead to a non-inflammatory hydrocephalus (Tyrrell and Cameron, 1957; Mims, 1960; Johnson and Johnson, 1972). Early studies describing the incomplete replication of A/WS/33 and A/PR/8/34 in mouse-brain showed a single cycle increase in haemagglutinin (HA) and neuraminidase (NA) activity, and nucleoprotein (NP)-associated antigen (Schlesinger, 1950; 1953). However, only a small proportion of viable virions were produced and these lacked a cleaved HA (Lazarowitz and Choppin, 1975; Klenk *et al*, 1975). Although a minority of these could be activated by trypsin, this still represents a vastly smaller amount than obtained with a neurovirulent strain (Schlesinger *et al*, 1989).

As a model for the abortive infection, replication of the non-neurovirulent strains A/PR/8/34 and A/WS/33 has been studied in detail in mouse embryo brain (MEB) cell cultures, which are established from embryos of 17 to 20 day gestation. Six days of incubation produces 'young' MEB cultures, representing undifferentiated cells, which support comparable replication of both neurovirulent and non-neurovirulent strains. 'Aged' MEB cultures, on the other hand, representing differentiated $\geqslant$21-day old cultures, and containing predominantly astrocytes, with some oligodendrites and neurons, as well as a few fibroblasts, do not. While neurovirulent strains can permissively infect the astrocytes, oligodendrites and neurons, the non-neurovirulent strains only show signs of replication in oligodendrites and neurons, and even here fail to induce cytopathic effects (Bradshaw *et al*, 1989a; 1990).

Other work on these non-neurovirulent strains has focused on the matrix (M) and non-structural (NS) genes. These genes encode proteins translated from either full-length transcripts, M_1 and NS_1 respectively, or spliced mRNA species, M_2 and NS_2 respectively (Lamb, 1989). For both the A/PR/8/34 and A/WS/33 strains, expression of M_1 and NS_1 was reduced, while M_2 and NS_2 were produced at normal levels or higher levels compared to those typical of a productive infection (Bradshaw *et al*, 1989b; 1990). This altered expression was due to an increased ratio of spliced to unspliced M and NS mRNA. The increased splicing was found to be related to the level of differentiation of the cells, with less differentiated cell types showing reduced splicing (Bradshaw *et al*, 1989b; 1990). This is similar to that observed in particular cell lines in which influenza virus shows abortive infection (Lohmeyer *et al*, 1979; Conti *et al*, 1980; Smith

and Hay, 1982; Inglis and Brown, 1984; Giesendorf *et al*, 1984). In addition, there was a lack of spontaneous HA cleavage, which would explain the requirement for trypsin to activate the small number of viable virions produced (Schlesinger *et al*, 1989). This study also found that the availability of the M_1 protein is a factor influencing the rate or extent of assembly of potentially infectious virions, and in this respect neurovirulent strains differed quantitatively rather than qualitatively.

Adaptation of influenza to mouse brain

The first isolated human influenza A virus strain, A/WS/33 (Smith *et al*, 1933), was successfully adapted to mouse brain to produce two neurovirulent variants, A/NWS/33 (NWS) (Stuart-Harris, 1939) and A/WSN/33 (WSN) (Francis and Moore, 1940). These variants undergo multiple cycles of replication in the brains of adult and neonatal mice and cause fatal encephalitis (Burnet, 1951; Tyrrell and Cameron, 1957; Fraser *et al*, 1959; Miyoshi *et al*, 1973). Both strains are also pneumovirulent for mice as a result of earlier propagation in mouse lung (Stuart-Harris, 1939; Francis and Moore, 1940), while the NWS strain is capable of causing a generalised viraemia, including neuropathy, in mice infected via the natural route (Wagner, 1955).

Apart from NWS and WSN, which were specifically adapted to mouse brain, and reassortants thereof, a limited number of other laboratory-derived stains also show neurovirulence even though they were not experimentally adapted. These include particular reassortants of the A/FPV/34 (FPV) strain (Vallbracht *et al*, 1979), a laboratory variant of A/Seal/Mass/1/80 called SC35, and further derivatives of this variant (Scheiblauer *et al*, 1995). Studies of these strains has provided additional information on the determinants of neurovirulence.

Interestingly, the NWS and WSN variants are more broadly cytopathogenic than non-neurovirulent strains. WSN produces cytopathic effects in differentiated human skeletal muscle cells (Armstrong *et al*, 1978), in a neuroblastoma cell line (Nakajima and Sugiura, 1980), in organotypic cultures of embryonic mouse hypothalamus (Gamboa *et al*, 1974) and ependymal organ cultures (Kohn *et al*, 1981). Similarly, NWS replicates with cytopathic effect in human diploid cell lines (Kilbourne *et al*, 1964; Ghendon *et al*, 1979). In addition, the FPV strain is also highly virulent, and able to cause a generalised infection of chickens, including the brain (Jungherr *et al*, 1946), while its highly neurovirulent recombinations produce a generalised viraemia in mice (Vallbracht *et al*, 1980). Similarly, the neurotropic SC35 variant of A/Seal/Mass/1/80 also causes systemic infection in mice and chickens and has gained the ability to infect MEB cell lines (Scheiblauer *et al*, 1995). This suggests that certain of the changes required for neurovirulence can act as general virulence determinants. In addition, the chick brain-virulent strain FPV has been successfully adapted to mouse brain by serial passage, suggesting that mammalian and avian neurovirulence determinants may be similar (Schäfer, 1955).

Molecular basis of neurovirulence

The variants NWS and WSN have acquired mutations in the genes which control virulence for mouse brain. However, given problems of strain variability, a number of levels of critical analysis have been utilised in order to associate a particular change with neurovirulence. These have included: (i) determination of the genes involved by reassortment; (ii) confirmation of sequences from different isolates, direct sequencing, etc; (iii) analysis of both of these independent neurovirulent derivatives; (iv) comparison of changes to other influenza sequences to assess normal variability at these sites; and (v) biological confirmation of predicted effects, for example, analysis of differences in mRNA splicing. By the use of this approach, we can have confidence in the importance of these changes to neurovirulence.

Genes responsible for neurovirulence

By analysis of reassortants from crosses between WSN and the non-neurovirulent strain A/Aichi/2/68, Sugiura and Ueda (1980) have shown that the NA, M and NS genes from WSN were required to express full neurovirulence in adult mice. The same genes were needed for efficient replication in a neuroblastoma cell line (Nakajima and Sugiura, 1980). However, since reassortants lacking the WSN NA gene were not able to multiply in mouse brain, it is likely that the M and NS genes function as accessory virulence factors to enable efficient replication, while the NA is the major neurovirulence determinant (Sugiura and Ueda, 1980). The NA gene has also been implicated as the major neurovirulence determinant for NWS (reviewed in Sugiura, 1975), with other genes implicated as accessory factors (Hobson *et al*, 1968; Mayer *et al*, 1973; Sugiura, 1975). However, unlike WSN, reassortants of the NWS strain can circumvent the requirement for the NA and retain neurovirulence if they possess the HA of the neurovirulent parent (Mayer *et al*, 1973).

Studies of the neurovirulent reassortants of FPV have revealed the HA and M genes of the FPV strain are important for expression of neurovirulence (Scholtissek *et al*, 1979; Vallbracht *et al*, 1979; 1980). In addition, certain polymerase genes, and occassionally the NP gene, are required from non-neurovirulent strain, probably reflecting a gene-constellation effect in this system (Scholtissek *et al*, 1979; Vallbracht *et al*, 1980; Bonin and Scholtissek,

1983). In one set of recombinants, the NS gene also contributed to neurovirulence (Bonin and Scholtissek, 1983). Changes in the HA gene were also implicated in the acquisition of neurovirulence of the SC35 variant of A/Seal/Mass/1/80, although subsequent derivatives which showed increased neurovirulence had no further changes in the HA, again suggesting other genes can contribute to neurovirulence (Scheiblauer *et al*, 1995).

Role of the NA gene

The NA gene encodes the enzymatically active glycosylated surface protein neuraminidase, which is able to catalyse the cleavage of the α-ketosidic linkages between the terminal sialic acid residue and the adjacent residue on the carbohydrate chain (Gottschalk, 1957). While the NA may be involved in viral penetration to respiratory epithelial cells by its action on mucus (Burnet *et al*, 1947; Burnet, 1948), it also removes receptors from the HA to prevent self-aggregation and to allow release of virus from the cell (Seto and Rott, 1966; Palese *et al*, 1974). While it is not clear how the NA contributes to neurovirulence, an important factor is its role of indirectly facilitating cleavage activation of the viral HA in cells which normally fail to cleave the HA (Schulman and Palese, 1977; Ghendon *et al*, 1979; Nakajima and Sugiura, 1980). The importance of HA cleavage for neurovirulence is demonstrated by the analysis of reassortants between WSN and A/Aichi/2/68, in which the presence of the WSN NA allowed replication both in mouse brain (Sugiura and Ueda, 1980) and in a neuroblastoma cell line (Nakajima and Sugiura, 1980), with the HA cleavage shown to be important for replication in both systems (Nakajima and Sugiura, 1980; Schlesinger *et al*, 1989). In other cell types as well the NA of the neurovirulent strains promotes HA cleavage. For example, the NWS NA promotes HA cleavage in human embyro fibroblast (HEF) cells and allows infectious virus to be produced while the A/WS/33 NA is incapable of facilitating HA cleavage and so infectious virus is not produced (Ghendon *et al*, 1979). Plaque formation of WSN in MDBK cells is also a result of the NA promoting cleavage of the HA (Schulman and Palese, 1977).

Sequence analysis of the NAs from the neurovirulent strains NWS and WSN has revealed five changes in common relative to their parent, A/WS/33 (Ward, 1995a). Comparison to the three-dimensional structure and other available sequences identified three positions – 130, 133 and 414 – as likely candidates for neurovirulence. These lie close to the active site and are at otherwise highly conserved positions (Ward, 1995a). Site-directed mutagenesis on the WSN NA gene using reverse genetics has provided decisive evidence of the carbohydrate at Asn^{130} in neurovirulence. The glycosylation site was reintroduced into the WSN sequence and the resultant strain was no longer neurovirulent. Two revertant viruses recovered were found to possess changes which again removed the glycosylation site at position 130 (Li *et al*, 1993). Therefore, the absence of a carbohydrate moiety at this site is one necessary condition for expression of neurovirulence. However, since the mutagenesis was performed on a WSN background, the importance of the other changes in the manifestation of neurovirulence cannot be discounted.

The non-neurovirulent glycosylation site mutant of WSN showed decreased activity on large substrates compared to WSN (Li *et al*, 1993), which the authors infer might be important for neurovirulence. However, the NWS NA is less able to cleave large substrates than A/WS/33 (Ward, 1995a) which is contrary to this argument. Likewise, analysis of the NA enzymes from the neurovirulent strains and A/WS/33 indicated no correlation between neurovirulence and either overall activity or preference for particular *N*-substitutions (Hobson *et al*, 1968; Ward, 1995a). In contrast, both NWS and WSN showed an increased preference for small substrates compared to A/WS/33 (Ward, 1995a), as did WSN compared to the glycosylation site mutant (Li *et al*, 1993). In addition, the enzymes from both neurovirulent strains showed an increased preference for substrates with 2→3 linkages, and their activity was potentiated by Ca^{2+} ions (Ward, 1995a).

So what function does the NA perform which facilitates HA cleavage? Li *et al.* (1993) argued that the NA of the neurovirulent strains is able to remove particular sialic acids from the HA allowing greater accessibility of host proteases to cleave the HA. However, if a specific carbohydrate on the HA is responsible for blocking access then it is surprising that HA mutants which had lost the site for this carbohydrate are not isolated instead of NA mutants, since there are many examples of the removal of specific carbohydrates from the HA correlating with increased virulence (Kawaoka *et al*, 1984; Anders *et al*, 1990; Ward and de Koning-Ward, 1995). Other possibilities, therefore, should be considered. For example, the NA may be able to cleave particular sialic acids from a host protease which then allows it to access the HA. Also, why must the Asn^{130} glycosylation site be removed? Perhaps this (and other?) changes in the NA lead to an altered specificity which allows novel sialic acid residues to be cleaved. Alternatively, specific lectins might bind to the carbohydrate at Asn^{130} in mouse brain and, since such lectin binding can sterically inhibit NA function (Palese *et al*, 1974), the loss of this glycosylation site might therefore be able to overcome the inhibition.

Role of the M gene

The M gene encodes two viral structural proteins, M_1 and M_2. The M_1 protein lines the inner layer of the viral membrane such that it contacts both the

internal ribonucleoprotein (RNP) complex and the surface glycoproteins (Ruigrok *et al*, 1989b). The M_1 protein binds lipid (amino acids 62–68 and 114–133) (Gregoriades and Frangione, 1981), RNA/RNP [residues 90–108 and 128–164 (Ye *et al*, 1987; 1989), or 70–140 (Hankins *et al*, 1989)], and other M_1 proteins to form oligomers (Gregoriades and Frangione, 1981). During infection the binding to nuclear RNP promotes export to the cytoplasmic membrane and virus assembly (Martin and Helenius, 1991; Helenius, 1992). The membrane-associated M_2 protein protects the structural integrity of the acid-sensitive HA by modulating the effect of low pH encountered in the *trans*-Golgi (Sugrue *et al*, 1990). The M gene has been implicated as an accessory virulence determinant for mouse brain (Sugiura and Ueda, 1980), with the M_1 protein identified as the rate-limiting step of virus assembly (Lazarowitz *et al*, 1971; Schlesinger *et al*, 1989).

To identify the changes responsible for the mouse brain virulence capabilities of the M gene, the sequence of the A/WS/33 M gene was compared to those of the mouse-brain adapted variants NWS and WSN (Ward, 1995b). The nucleotide substitutions in the neurovirulent variants only affect the M_1 protein. There is an Ala^{41}→Val substitution in both NWS and WSN, as well as Thr^{139}→Ala and Ala^{227}→Thr changes only in NWS. Examination of M_1 sequences of other strains identified a correlation between the first two of these changes and increased virulence for mouse lung (Ward, 1995b). Thus, the acquisition of at least one of these specific amino acid substitution in the M_1 protein (Ala^{41}→Val and/or Thr^{139}→Ala) appears important for full expression of neurovirulence.

The change at position 41 has been correlated with the acquisition of resistance to a monoclonal antibody to the M_2 protein (Zebedee and Lamb, 1989a). The results of this study imply that the M_1 change may compensate for a loss of M_2 function caused by the presence of the antibody, such as during the passage of the virus through the acidic endosome after endocytosis, where the M_2 protein seems to play an important role. 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} (Yasuda *et al*, 1993b). Similarly the change at position 139 is in the region responsible for RNA/RNP binding (Ye *et al*, 1987; 1989) and so may affect this same process. Indeed it has been suggested that the Thr^{139}→Ala change may affect the pH-dependent association/dissociation of M_1 with RNP to control virulence and growth (Smeenk and Brown, 1994). It seems likely that the Ala^{41}→Val change may also affect growth since the WSN M gene has been linked to increased growth rate in eggs (Klimov *et al*, 1991) and MDCK cells (Yasuda *et al*, 1993b), while the A/PR/8/34 M gene (which also possesses the Val^{41}) has also been implicated as a determinant of *in ovo* growth rate (Baez *et al*, 1980). Further investigations into this potentially common mechanism of increasing virulence and growth seem warranted. These changes, however, have been shown to be present in mouse-lung adapted variants of influenza A virus, with the Thr^{139}→Ala change directly shown to control virulence for mouse lung (Smeenk and Brown, 1994). Therefore, the specific amino acid substitutions in the M_1 protein would seem to correlate with adaptation to mouse tissue in general rather than specifically to mouse brain (AC Ward and EM Anders, manuscript in preparation).

As discussed earlier, increased splicing of this gene, with a concomitant decrease in M_1 levels, was observed in the non-neurovirulent strains A/PR/8/34 and A/WS/33, while WSN showed decreased levels of splicing and increased levels of M_1 protein. In addition, NWS showed decreased M gene splicing relative to A/WS/33 (Ward *et al*, 1995a). Interestingly, there were no other changes in the M gene to account for these altered splicing levels (Ward, 1995b). This suggests that splicing of this gene may be regulated in *trans*, consistent with other studies (Lamb and Lai, 1982; Valcárel *et al*, 1993). The importance of the NS gene in this regard is discussed below.

Role of the NS gene

The NS gene encodes two proteins, NS_1 and NS_2. The synthesis of NS_1 is essential for the normal replication of vRNA (Hatada *et al*, 1990), it interacts directly with vRNA (Hatada *et al*, 1992), and has been shown to increase translation of specific viral mRNAs, particularly the M_1 message (Enami *et al*, 1994). The NS_2 protein interacts directly with the M_1 protein (Yasuda *et al*, 1993a; Ward *et al*, 1995b) and has been shown to be present in the influenza virus particle (Richardson and Akkina, 1991; Ward *et al*, 1995b). A mutant NS_2 facilitates aberrant replication of one of the polymerase genes (Odagiri and Tobita, 1990), implying a role for the protein in viral packaging.

As eluded to earlier, A/WS/33 showed restricted expression of NS_1 and increased expression of NS_2 in differentiated MEB culture relative to undifferentiated cells, which is due to increased splicing of the NS gene (Bradshaw *et al*, 1989b; 1990). In contrast, the levels of splicing of the NS gene remain unchanged with the neurovirulent derivative WSN, which allowed levels of NS_1 protein to remain high (Bradshaw *et al*, 1989b; 1990). Sequence comparison of the NS genes of the neurovirulent strains relative to their parent revealed changes around the 3′ splice-site which serve to increase the thermal stability of this region, and to sequester the branch-point A residue in secondary structure (Ward *et al*, 1993; 1995a). This was correlated with decreased splicing of both NS and M genes (Ward *et al*, 1995a). Therefore, the masking of the 3′ splice-site in

neurovirulent strains can counteract the inherent increase in splicing of the NS gene in mouse brain. Thus, the levels of spliced NS mRNA in the mouse brain can return to those commensurate with a productive infection of the neurovirulent strains. In this regard, the changes in M gene splicing are likely to be a result of the increased expression of the NS_1 protein seen in WSN (Bradshaw *et al*, 1990) and NWS (AC Ward, unpublished) as a result of the reduction in NS gene splicing, since the NS_1 protein controls splicing of the M gene (Hatada *et al*, 1990; Snyder *et al*, 1990). Therefore, the increased M_1 protein seen in neurovirulent strains can be explained by both the decreased splicing of the M gene and stimulation of M_1 mRNA translation, also attributable to the increase in NS_1 (Enami *et al*, 1994).

Thus, the contribution of the NS gene to neurovirulence is at the level of mRNA splicing, directly controlling the relative levels of NS_1 and NS_2, and indirectly the levels of M_1 and M_2 protein. It is likely the indirect effect may be the most important, since the levels of M_1 protein is the rate-limiting step of viral replication (Schlesinger *et al*, 1989), although a role for the increased levels of NS_1 in cytopathology cannot be discounted (Bradshaw *et al*, 1990). In this way, the changes in the NS gene can act in concert with other changes to allow effective replication in the mouse brain.

Role of 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 (Wilson *et al*, 1981; Rogers *et al*, 1983; Weis *et al*, 1988). In its cleaved form the HA consists of two disulphide linked subunits which are non-covalently associated as an HA trimer (Wharton *et al*, 1989). Cleavage of the HA is essential for infectivity of the virus particle (Lazarowitz and Choppin, 1975; Klenk *et al*, 1975). 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 (Wiley and Skehel, 1987; Ruigrok *et al*, 1989a). The importance of HA cleavage for neurovirulence is demonstrated by the analysis of reassortants between WSN and A/Aichi/2/68, in which the presence of the WSN NA allowed replication both in mouse brain (Sugiura and Ueda, 1980) and in a neuroblastoma cell line (Nakajima and Sugiura, 1980) through facilitating HA cleavage. The HA of the NWS strain is sufficient for neurovirulence, since it can dispense with its NA and retain neurovirulence (Mayer *et al*, 1973). This is presumably because the NWS HA is highly susceptible to cleavage in a wide variety of systems (reviewed in Kilbourne, 1963) which obviates the absolute need for the NA to facilitate its cleavage. In support of this, the highly cleavable HA derived from A/FPV/Rostock/34 is also essential for the neurovirulence of progeny reassortants (Scholtissek *et al*, 1979; Bonin and Scholtissek, 1983), while the SC35 variant of A/Seal/Mass/1/80 which is capable of replication in mouse brain also possesses a highly cleavable HA (Li *et al*, 1990). However, HA cleavage is not the only determining factor in virus replication; for example, WSN undergoes abortive infection in HeLa cells despite HA cleavage occurring (Gujuluva *et al*, 1994).

Sequence analysis has revealed that the virulence capabilities of the NWS HA may involve at least three different mechanisms: (i) loss of a potential glycosylation site; (ii) a change at the cleavage site; and (iii) a substitution in HA_2 which may increase the pH of fusion (Ward and de Koning-Ward, 1995). The potential glycosylation site lost is at Asn^{162}, in the vicinity of the receptor binding site. Therefore, one mechanism to explain the increased virulence of NWS is via a decreased sensitivity to lectins, such as β inhibitor, a component of normal serum which binds mannose (Hartley *et al*, 1992), resistance to which has been shown to accompany adaptation to mouse lung (Brown, 1990; Gitelman *et al*, 1986). In support of this, the equivalent glycosylation site is lost in the bovine serum-resistant variants of $Mem71_H$-Bel_N (Anders *et al*, 1990) and A/Phil/82 (Hartley *et al*, 1992) which have both gained resistance to mouse β inhibitor. Loss of this site is also seen in the mouse-adapted strains A/PR/8/34 (Winter *et al*, 1981) and WSN (Hiti *et al*, 1981), both of which are resistant to guinea-pig β inhibitor (Yamamoto *et al*, 1987), which has similar properties to mouse β inhibitor (Anders *et al*, 1994). Other studies have also linked mouse-adaptation with loss of glycosylation sites. For example, studies with the H3N2 reassortant 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 mouse lung (Hartley *et al*, 1992; CA Hartley, PC Reading, AC Ward and EM Anders, manuscript submitted), while the mouse lung-adaptation of the H1 strain A/USSR/77 showed two successive amino acid substitutions, with loss of glycosylation sites at residues 87 and 127 (94a and 131 in H3 numbering), both in vicinity of receptor-binding site (Gitelman *et al*, 1986; Kaverin *et al*, 1989). Thus, loss of glycosylation sites from the HA may represent a common mechanism for mouse-adaptation (AC Ward and EM Anders, manuscript in preparation).

The NWS strain also has a unique $Ser^{326} \rightarrow Phe$ change at the residue immediately preceeding the Arg-Gly cleavage site between HA_1 and HA_2 (Ward and de Koning-Ward, 1995). Only the WSN strain has a large hydrophobic residue at this position (Hiti *et al*, 1981), and although the HA of WSN has not been implicated in neurovirulence it is, like the

NWS HA, highly cleavable in a number of systems (Lazarowitz and Choppin, 1975). Therefore, to explain the increased cleavability of the NWS and WSN HAs, it could be argued that the amino acid change at the cleavage site allows a protease with specificity for a large hydrophobic residue at the −2 position to cleave the HA of these strains. A plasmin-like enzyme is a good candidate because the WSN HA can undergo cleavage activation by plasmin, unlike other strains tested, obviating the need for a trypsin-like enzyme wich is normally required (Lazarowitz *et al*, 1973; Lazarowitz and Choppin, 1975). As a corollary, the HA of the FPV strain can circumvent the need for a trypsin-like enzyme for initial cleavage by the presence of the basic stretch amino acids at the cleavage site (Bosch *et al*, 1981) which can be recognised by furin or furin-like proteases (Stieneke-Gröber *et al*, 1992). Similarly, the SC35 variant shows increased HA cleavage as a result of the insertion of three arginine residues adjacent to the HA cleavage site (Li *et al*, 1990).

In addition, the NWS HA gene has a $Asp^{112} \rightarrow Val$ change in the HA_2, a residue almost completely conserved in field strains (Ward and de Koning-Ward, 1995). Non-conservative changes at this site in experimentally-derived strains have been correlated with an increased pH optimum of HA-mediated endosome fusion (Daniels *et al*, 1985). Similarly, the single amino acid change in HA_2 ($Gly^{47} \rightarrow Trp$) which results in increased virulence of the mouse-adapted variant of A/FM/1/47 is also at such a site (Smeenk and Brown, 1994). By analogy, variants of influenza selected for their ability to grow in MDCK cells have an elevated fusion pH threshold (Rott *et al*, 1984). Alterations in the pH of fusion may, therefore, represent a general pathway to increased virulence, and so a likely mechanism to explain at least some of the increased virulence of the NWS strain.

Biology of neurovirulence

Replication of non-adapted strains in mouse brain
The sites where the replication of non-adapted strain (such as A/WS/33) in the mouse brain is deficient, or liable to inhibition, are presented schematically in Figure 1. The HA of the A/WS/33 strain is largely uncleaved in this tissue (Schlesinger *et al*, 1989), and so fusion of the virus is blocked. In addition, low levels of M_1 observed for this strain affects assembly, since the availability of the M_1 protein determines both the extent and rate of assembly of potentially infectious progeny virions in mouse brains (Schlesinger *et al*, 1989). The low levels of M_1 protein observed during infection of mouse brain with A/WS/33 presumably result from the reduced levels of the NS_1 protein (Bradshaw *et al*, 1989a; 1990), the production of which has been shown to stimulate M_1 mRNA translation

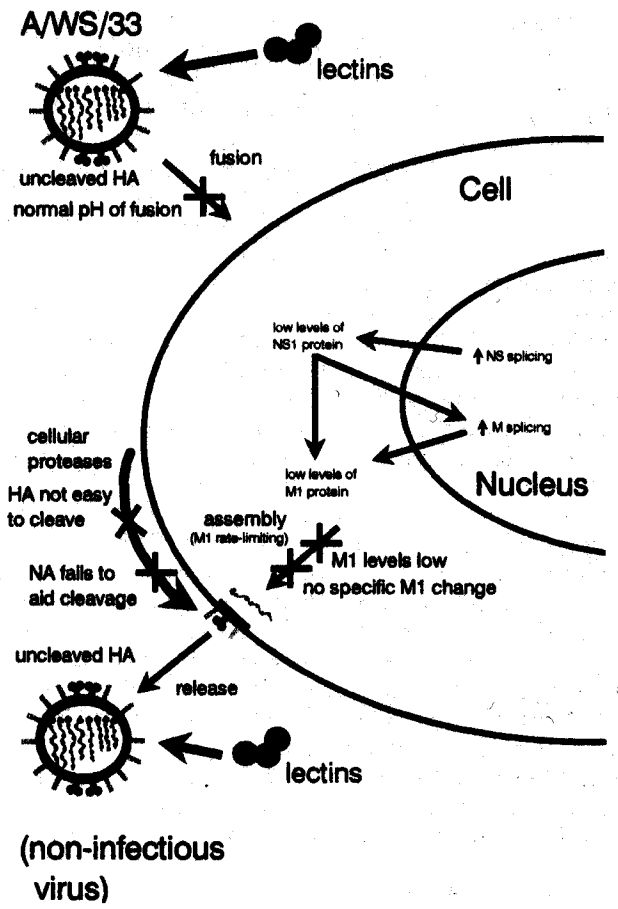

Figure 1 Abortive replication of A/WS/33 in mouse brain.

(Enami *et al*, 1994), as well as to inhibit splicing of the M gene (Hatada *et al*, 1990). For those A/WS/33 virus particles which are released, the HA is not easily cleaved (Schlesinger *et al*, 1989), and the NA cannot facilitate HA cleavage (Ghendon *et al*, 1979). Therefore, the particles have a predominantly uncleaved HA and so are largely non-infectious. In addition, the loss of carbohydrate from its neurovirulent derivatives suggests that the action of lectins on this strain might be important in blocking viral infectivity.

Replication of adapted strains in mouse brain
After consideration of the impediments to replication of A/WS/33 in mouse brain, the possible effects of the observed changes in the neurovirulent strains can be evaluated. Firstly, there were a number of changes evident in the mouse brain-adapted strains which are also seen in variants adapted to mouse lung: (i) specific changes in the M_1 protein ($Ala^{41} \rightarrow$ Val and/or $Thr^{139} \rightarrow$ Ala); (ii) alterations in the HA at sites associated with increased pH of fusion; (iii) loss of a specific glycosylation site in the HA_1. Possible effects of these changes in facilitating a productive infection of the NWS strain are shown schematically

in Figure 2. Changes (i) and (ii) are both associated with phenotypes related to endosomal pH: the former change correlating with the M_1 protein dissociating from RNPs at a higher pH (Yasuda *et al*, 1993b), the latter associated with a change in the pH of virus-cell fusion (Wharton *et al*, 1989). Change (iii) suggests that combating lectins is an important determinant of virulence, since changes at this site are associated with resistance to β inhibitor (Hartley *et al*, 1992; Kaverin *et al*, 1989).

The presence of change (i) with either of change (ii) or (iii) would appear sufficient for mouse lung adaptation. This is supported by studies on the mouse lung adaptation of A/FM/1/47 (Smeenk and Brown, 1994) and Phil82/BS (CA Hartley, PC Reading, AC Ward and EM Anders, manuscript submitted) and is consistent with the limited studies on mouse lung-adapted A/Port Chalmers/1/73 (Zebedee and Lamb, 1989b). Therefore, the adaptation of influenza to mouse lung appears to require just a subset of those changes required for adaptation to mouse brain. Thus, the NWS and WSN strains are virulent for mouse lung as well as mouse brain, while all highly neurovirulent FPV recombinants are pneumovirulent, although pneumovirulent recombinants are not necessarily neurovirulent (Vallbracht *et al*, 1979). This lends support to the concept of a hierarchy of virulence determinants for different mouse tissues.

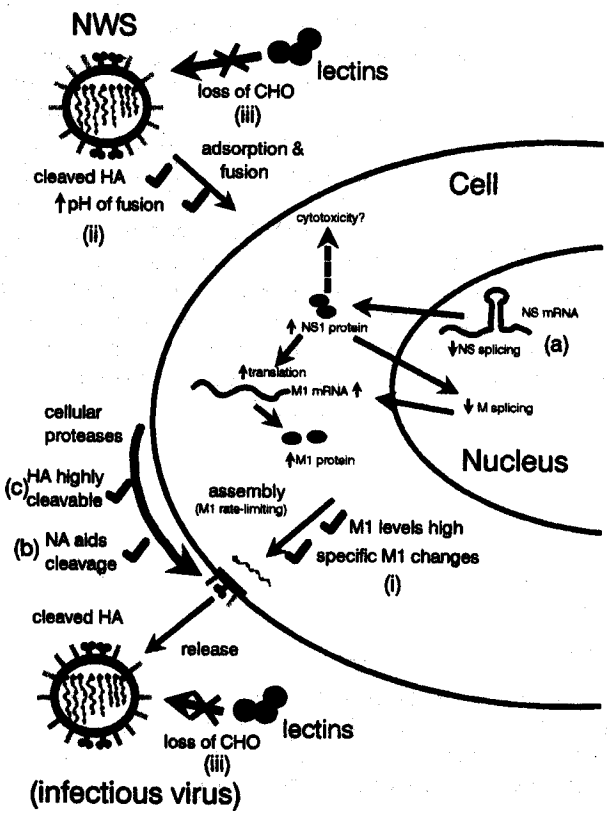

Figure 2 Permissive replication of NWS in mouse brain.

For adaptation to mouse brain, a number of specific changes are observed in addition to those needed for adaptation to mouse lung: (a) increased secondary structure at the 3′ splice-site of the NS gene, which inhibits splicing of this gene; (b) changes in the NA (likely to be the loss of the glycosylation site at position 130) which facilitate HA cleavage; (c) changes affecting the HA cleavage site iself. The effects of these changes are shown schematically in Figure 2. Change (a) seems necessary to compensate for increased splicing of the NS gene transcript in neural tissue (Bradshaw *et al*, 1989b). In addition, since this change leads to increased levels of NS_1 protein, it can influence the levels of M_1 protein by inhibiting splicing (Hatada *et al*, 1992) and promoting translation (Enami *et al*, 1994) of the M_1 mRNA. Changes (b) and (c) both serve to increase cleavage of the HA polypeptide and allow infectious virions to be released. This is presumably required to compensate for the reduced cleavage of the HA observed in mouse brain for non-adapted strains (Schlesinger *et al*, 1989).

Not all of the changes are absolutely required for neurovirulence. Change (a) is seen in both neurovirulent variants. However, the NS gene is only an accessory determinant of virulence and so this change is not absolutely required to express neurovirulence (Sugiura and Ueda, 1980). In addition, only one of changes (b) and (c) appears necessary, since reassortants of the WSN strain only require the NA gene from the neurovirulent strain to express neurovirulence (Sugiura and Ueda, 1980), while reassortants of NWS can remain neurovirulent as a result of expressing either the NWS HA or NA (Mayer *et al*, 1973), as either will allow for a cleaved HA in the progeny. The importance of HA cleavage for neurovirulence is further supported by observations that pneumovirulent strains can be made neurovirulent by the presence of the highly cleavable HA of the A/FPV/Rostock/34 strain (Vallbracht *et al*, 1980).

Access to the CNS

All of the changes discussed above relate to effects on replication. It is also pertinent to consider accesses of the virus to the CNS, whether by immunologically mediated injury or a disrupted blood-brain barrier. However, while the exact mechanism remains unknown, this does not appear to play an important role in determining neurovirulence, since Factor VIII positive capillary endothelial cells from adult mouse brains are equally permissive to infection with adapted and non-adapted strains (Bradshaw *et al*, 1989b).

Future directions

The studies into neurovirulence have thus far provided a great insight into both the biology and

Table 1 Changes associated with neurovirulence

Gene	*Changes observed*	*Effects on replication*
NA	Asn^{130} glycosylation site lost (WSN & NWS) Val^{133} mutation (WSN & NWS) Arg^{414} mutation (WSN & NWS)	• altered substrate specificity, with preference for small substrates and 2→3 linkages
M	M_1 Ala^{41}→Val (WSN & NWS)* M_1 Thr^{139}→Ala (NWS)*	•↑ growth • altered association/dissociation properties of M_1-RNP complexes
NS	↑ secondary structure at 3′ splice site (WSN & NWS)	•↓ splicing of NS (& M) gene
HA	HA_1 Asn^{162} glycosylation site lost (WSN & NWS)* HA_1 Ser^{326} mutation (WSN & NWS) HA_2 Asp^{112} mutation (NWS)*	•↓ lectin sensitivity? •↑ HA cleavage • altered pH of fusion

* Also observed in mouse lung-adapted strains

molecular basis of neurovirulence (summarised in Table 1). The next step is to examine these changes in isolation, in order to determine their relative importance, particularly with regard to their role in neurovirulence *per se* compared to their effects on growth rate and virulence generally. The most appropriate means to address this is the use of 'reverse genetics' to create isogenic strains differing only by single changes, an approach used to look at the NA gene (Li *et al*, 1993). This is important since genes may behave differently in different genetic backgrounds, a phenomenon known as a 'gene constellation effect' (Rott *et al*, 1984). The results summarised in this review also provide a relevant set of parameters with which to examine epidemic virus strains which have been associated with elevated CNS symptoms in humans. In addition, there is an obvious need to investigate more closely the mechanisms of CNS entry.

Neurovirulence and other viruses

Neurovirulent derivatives of a number of other viruses have also been generated, and their study has provided uncanny parallels for many of the key concepts discussed in this review. For example, single amino acid changes in the envelope glycoprotein of Sindbis virus can lead to neurovirulence (Levine and Griffin, 1993), while a single amino acid position in the glycoprotein determines neurotropism of lymphocytic choriomeningitis virus (Villarete *et al*, 1994). Similarly, either of two single amino acid substitutions in the envelope glycoprotein of Dengue virus (one of which represents the loss of glycosylation site) can increase neurovirulence (Kawano *et al*, 1993). In addition, infection of mouse brain with measles or vesicular stomatitis virus is characterised by restricted expression of M proteins (Kristensson and Norrby, 1986).

Conclusions

Mouse neurovirulence represents a useful *in vitro* model for human CNS complications. While it is a somewhat artifical system, the studies presented in this review bring us to a closer understanding of the molecular changes required for influenza virus to propagate in an atypical host. The results provide direct support that virulence is modular, with more than one virulence determinant able to be found both in the same gene, and on separate genes in the same virus. These virulence determinants show some degree of specificity, with certain subsets important in the adaptation of influenza to a particular cellular milieus, although there is a general improvement in replication in many cells. In addition, most of the specific changes are single amino acid substitutions (which are the result of single nucleotide mutations) and so the process of adaptation shows clear parallels to 'antigenic drift'. Since the replication machinery of influenza has an inherently high mutation rate, the virus is geared to exploit both adaptation and antigenic change to display both a wide host range and continued prevalence in particular hosts. Such continued subversion of cellular defence mechanisms further attests to the chameleon nature of the virus and its importance in viral evolution.

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