Commentary

Viral Tropism and the Pathogenesis of Influenza in the Mammalian Host

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Influenza viruses, single-stranded negative-sense RNA viruses within the family *Orthomyxoviridae*, remain an important cause of human morbidity and mortality worldwide. Despite effective vaccines, more than 200,000 individuals in the United States are hospitalized each year for influenza, and approximately 35,000 succumb to disease.¹ Seasonal epidemics arise through antigenic variation and recombination events from existing and currently circulating human strains, which initially target the upper respiratory tract with extension to a bronchointerstitial pneumonia in a subset of affected individuals. Disease is often more severe in older and debilitated patients, producing a characteristic distribution in age-related mortality within the affected population.²

In contrast to seasonal epidemics, pandemics arise when antigenically novel viruses emerge and are readily transmissible within the naïve human population. Such events are rare and occur after zoonotic transmission of viruses through recombination events between established human and avian strains or possibly through direct adaptation of avian strains for efficient human to human transmission.³ Concerns over an avian influenza pandemic have risen since 1997 with the recognition of H5N1 as a cause of fulminant disease in humans.^{4,5} Pathologically fatal H5N1 causes diffuse alveolar damage and progression to multiple organ dysfunction with a case fatality rate estimated at more than 50%. Although H5N1 disease is severe, transmission rates have been low, and viral adaptation to efficient human to human transmission has not occurred.

Understanding viral and host barriers that prevent transmission may be critical in establishing rational control measures as well as predicting and stratifying risk for individual strains of influenza. In this issue of *The American Journal of Pathology*, van Riel et al⁶ examine the viral binding distribution in pulmonary tissue of a number of avian and human influenza strains to elucidate the role of tropism in transmission and pathogenesis.

Viral Targets As a Restriction Factor

Host restriction factors may prevent the spread of influenza viruses within the human population and likely play a role in preventing infection with novel avian strains and the establishment of pandemics. Viral elements that determine host restriction are thought to lie within the hemagglutinin (HA) and neuraminidase envelop proteins and within the nucleocapsid and polymerase basic 2 proteins.³ Traditionally, the HA protein is thought to play a key role in determining host specificity. Influenza viruses of human origin selectively bind to glycolipids or glycans that contain terminal sialyl-galactosyl residues with a 2-6 linkage (SA α -2,6), whereas avian viruses bind to sialyl-galactosyl residues with a 2-3 linkage (SA α -2,3).

The distribution of these host cellular receptors has historically been investigated by histochemical staining with lectins of known sialyl-galactosyl specificity. As defined by lectin histochemical staining, the distribution of sialic acid residues is thought to play a role in defining host specificity. SA α -2,6 moieties are found within the respiratory tree of humans and other mammals, and SA α -2,3 moieties are found in the gastrointestinal tract and pulmonary tree of avian species. Human influenza strains typically bind SA α -2,6 found primarily in the upper respiratory tract, and localization to this region is thought to increase the risk of infection and transmission between individuals. In contrast, the absence of SA α -2,3 residues from the upper respiratory tract of humans has been proposed as an important restriction factor. Thus differential expression of sialic acid residues may help explain the pathological consequences of human influenza infection and likely prevents cross-species or zoonotic transmission events of avian influenza strains to man.

Although lectin staining was initially a useful surrogate in explaining host specificity, this dichotomy is an over-simpli-

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fication of viral HA-host receptor interactions. Lectin histochemical staining is dependent on specific linkages within the sialyl-galactosyl residue, but glycans may be further modified, thereby affecting their affinity for HA. In addition to cell-associated sialyl-galactosyl residues, mucins also contain sialic acids that may bind to HA, preventing productive infection of cells. Recently, SA α -2,3 glycans, the principal receptor of avian strains, have been demonstrated in the lower respiratory tract of humans, localizing to type II pneumocytes and cuboidal nonciliated cells of the terminal airways.⁷ Thus, location of specific sialic acid moieties within the microenvironment of the respiratory tree may be critical in determining the outcome of exposure. Finally, ongoing viral mutations and adaptation may change host receptor affinity over time, further complicating interpretation of lectin staining.⁸ Although glycan expression likely affects viral binding and tropism, other host and viral factors contribute to host restriction.

Viral Tropism and Pathogenesis

To further investigate differential viral tropism, van Riel et al⁶ use an *in vitro* binding assay to examine the pattern of viral adherence (PVA) of a number of human and avian influenza viral strains to tissues from normal humans and laboratory animals. The assay relies on concentrated virus labeled with fluorescein, which is then incubated with normal tissue sections. Binding of the virus can then be detected by routine immunohistochemical techniques directed at the fluorescein label. The assay bypasses reliance on lectin specificity, which may not accurately predict subtleties of host viral interactions, and allows double-label experiments to be performed to definitively identify cell types. The authors expand on their previously published work examining the distribution of H5N1 binding on human tissue and here demonstrate differential adhesion patterns between avian and human strains.⁹ Two human influenza strains (H1N1 and H3N2) adhered more strongly to tracheal and bronchial tissue and thus corresponded with the primary lesion of tracheobronchitis observed with typical epidemic influenza. In contrast, avian strains (H5N1 and H6N1) adhered to type II pneumocytes and alveolar macrophages in the lower respiratory tract, a finding consistent with the pathogenic development of diffuse alveolar damage with H5N1. These findings have implications in understanding viral pathogenesis and selecting animal models with relevance to human disease.

In this study, the authors importantly show differential binding of avian H5 and H6 viruses to human alveolar macrophages, a finding that was not observed with H3 or H1 human influenza viruses. The alveolar macrophage may play a critical role in disease pathogenesis not through production of infectious virus but rather through the up-regulation of proinflammatory cytokines that may further damage alveolar pneumocytes and directly contribute to multiple organ dysfunction.¹⁰ The small number of autopsies performed on human patients with confirmed H5N1 has revealed the presence of a hemophagocytic syndrome as a factor that may have contributed to multiorgan dysfunction.^{5,11} The hemophagocytic syndrome has previously

been linked to excessive cytokine production, such as that observed in fulminant Epstein-Barr virus infection. This has led to the speculation that the pathogenesis leading to fatal outcome from H5N1 infection may differ from that seen with typical human influenza and the hypothesis that production of key proinflammatory cytokines may have a detrimental effect on disease progression. *In vitro* infection studies using H5N1 and human blood-derived monocyte/macrophages resulted in the marked induction of a number of monokines, including interferon- β and tumor necrosis factor- α , changes that were blunted when cells were similarly infected with the human influenza strains H1N1 and H3N2. Cytokine dysregulation is thought to be mediated through p38K mitogen-activated protein kinase pathways, and recombinant studies suggested that determinants of cytokine hyperinduction may partially reside within the NS gene encoding the NS1 and viral nuclear export proteins.^{10,12} The current findings by van Riel et al⁶ suggest that infection of alveolar macrophages may also be more efficient with avian strains and support a key role for these cells in the pathogenesis of fulminant avian influenza.

Inconsistencies in Viral Tropism Studies

Because of the fundamental importance of viral tropism on disease transmission and pathogenesis, there have been a number of recently published reports attempting to define viral distribution and targets, resulting in a range of sometimes conflicting results. Why might there be differences in viral distribution identified through use of the *in vitro* PVA binding assay, viral localization after natural or experimental infection, and *in vitro* infection of respiratory explant tissue cultures?

Comparison of PVA studies and *ex vivo* tissue infection experiments reveals some interesting differences. Although most studies have suggested that the target of H5N1 infection of humans is limited to the lower respiratory tract, two recent reports using *in vitro* inoculation of respiratory tissue explants have suggested infection of cells of the upper air ways.^{13,14} Furthermore, these reports suggest that human strains target primarily nonciliated epithelium and that avian strains target ciliated epithelium and thus are in stark contrast to PVA studies published here and elsewhere.^{7,9} The reasons for these differences are not known but may relate to inherent shortcomings with the respective assays. Although the PVA assay described by van Riel et al⁶ may accurately predict HA host receptor interactions, it does not take into account other host factors after entry that may block viral replication. In addition, fixation of tissue and labeled virus may adversely alter binding affinity, thereby reducing apparent tissue distribution. In contrast, procedures used in explant studies may wash off surface mucins that form an important *in vivo* barrier and expose or induce receptors that are not normally accessible to virions *in vivo*, thus expanding the apparent binding pattern of avian viruses. The patient history and exact anatomical source of resected tissue as well as strains and passage history of inoculated virus could all impact these types of assays

and make direct comparisons difficult. Clearly, further work will be required to resolve these discrepancies.

The PVA assay also differs in subtle ways from results observed after inoculation of experimental animals. Although the assay failed to demonstrate appreciable adhesion to macrophages in the cynomolgus macaque, in a previous article, the coauthors describe immunohistochemical localization of H5N1 nucleoprotein antigen within alveolar macrophages in animals 4 days after experimental inoculation.¹⁵ This suggests that macrophage activation may alter viral binding and that patterns of viral infection may broaden with disease progression. Viral infection may produce a positive feedback loop for recruitment and activation of macrophages through the up-regulation and release of monocyte chemoattractant protein-1 and tumor necrosis factor- α followed by subsequent viral infection of newly recruited and activated monocytes. Some caution is advised in overinterpreting PVA distribution as a static phenomenon, and further work to define host receptor expression during disease progression may give additional insight to pathogenesis.

Selection of Animal Models

A number of species have been used as potential experimental models of H5N1 infection of humans, including mice, ferrets, cats, pigs, and macaques.^{16–20} Subtle and significant differences in virulence, pulmonary and extrapulmonary pathology, and viral distribution exist between models, and the relevance of each to human disease is unclear. Findings by van Riel et al⁶ suggest the binding pattern to human tissue of avian influenza, including H5N1, is most similar to that observed in cats, ferrets, and pigs, corresponding to *in vivo* work indicating the most severe pulmonary lesions are found in the terminal bronchioles and alveoli.

Selection of an appropriate species is complex and requires consideration of the elements of the disease process to be modeled as well as availability of reagents, cost, and biosafety. The disease in ferrets and cats is severe with demonstrated dissemination to multiple organs, including the central nervous system, liver, and lymphoid tissue.^{17,21} Such widespread dissemination has not been recognized in the limited number of human autopsies performed on H5N1 patients, and whether these species represent appropriate models for the pulmonary disease and secondary multiple organ failure observed in man remains to be determined. Although viral tropism is one component that may impact transmission and pathogenesis, other factors, such as the host's inflammatory response to viral infection, may be critical in the development of fulminant pulmonary disease.

Future Directions and Needs

Much remains to be learned about the pathogenesis and relevance of viral tropism in human and avian viral influenza. Glycan microarray assays represent a novel technology that has recently been developed to examine

glycan protein binding interactions, and they may help clarify these matters.²² This work reveals the complex nature of HA host-receptor interactions and demonstrates differences in binding of viral strains to the SA α -2,3 and α -2,6 families of glycans and binding to fucosylated and sulfated glycolipids.²³ Coupled with other molecular techniques, glycan arrays have proved a powerful tool to define binding specificity of viral HA at the amino acid level. Full realization of this technology is currently hindered by a lack of detailed understanding of glycan expression on host target cells throughout the complete microenvironment of the respiratory tract during health and disease.

Expansion of tropism assays to examine additional human and animal tissue outside the respiratory tree would give insight to alternative routes of infection and the mechanisms of viral dissemination. For example, experimental and natural inoculation of felids by the oral route leads to viral infection and likely dissemination from the gastrointestinal tract.^{18,24} Viral localization studies performed on cats reveals H5N1 antigen within the submucosal and myenteric plexi.²¹ Likewise, the oral route of transmission has been suspected in human patients, and diarrhea is a common clinical sign during H5N1 infection.²⁵ Consumption of raw duck's blood has been identified as a risk factor for human disease, and H5N1 viral RNA has been detected in the gastrointestinal tract.^{25,26} The mechanism of viral entry is unknown, and PVA studies would help determine initial targets in both species. If oral inoculation proves a significant route in humans, this may have important implications in disease prevention and vaccine development.

The pathogenesis of fatal H5N1 infection involves a number of viral virulence factors and detrimental host responses resulting in diffuse alveolar damage and progressive multiorgan dysfunction. Differential targeting of specific cell types likely contributes to this process; however, determinants outside of HA undoubtedly also play a role in viral virulence and dissemination. There is a critical need for additional human autopsy data to be published to define further the role of viral determinants on disease pathogenesis. Although more than 300 patients with confirmed H5N1 have been recognized worldwide, autopsy results are limited to a handful of cases, and whether such cases are truly representative of the disease process is unclear.^{11,26–28} Patients often have a history of antiviral and other therapies that may alter or artificially prolong disease course. Nonetheless, these scattered reports do support the findings of van Riel et al⁶ that targeting of the lower airways, type II pneumocytes, and alveolar macrophages occurs in severe H5N1 disease and likely plays a critical role in pathogenesis. Examination of additional tissue from naturally occurring disease would greatly help clarify the role of viral tropism and other virulence factors in disease progression.

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