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Review

Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Humans

Christine Korteweg, M.D., and Jiang Gu, M.D., Ph.D.

From the Department of Pathology and Infectious Disease Center, School of Basic Medical Sciences, Peking (Beijing) University, Beijing, China

H5N1 avian influenza is a highly fatal infectious disease that could cause a potentially devastating pandemic if the H5N1 virus mutates into a form that spreads efficiently among humans. Recent findings have led to a basic understanding of cell and organ histopathology caused by the H5N1 virus. Here we review the pathology of H5N1 avian influenza reported in postmortem and clinical studies and discuss the key pathogenetic mechanisms. Specifically, the virus infects isolated pulmonary epithelial cells and causes diffuse alveolar damage and hemorrhage in the lungs of infected patients. In addition, the virus may infect other organs, including the trachea, the intestines, and the brain, and it may penetrate the placental barrier and infect the fetus. Dysregulation of cytokines and chemokines is likely to be one of the key mechanisms in the pathogenesis of H5N1 influenza. We also review the various molecular determinants of increased pathogenicity that have been identified in recent years and the role of avian and human influenza virus receptors in relation to the transmissibility of the H5N1 virus. A comprehensive appreciation of H5N1 influenza pathogenetic mechanisms should aid in the design of effective strategies for prevention, diagnosis, and treatment of this emerging disease.

H5N1 avian influenza was initially confined to poultry, but in recent years it has emerged as a highly fatal infectious disease in the human population. In 1997, the avian influenza A virus subtype H5N1 crossed the avian-human species barrier for the first time.[1] Eighteen individuals were infected, six of whom died.[2] In January 2003, avian influenza re-emerged among humans in Hong Kong,[3] and since 2004 numerous human infections have also occurred in other Asian and non-Asian countries. To date, the World Health Organization has reported 348 laboratory-confirmed cases, 216 of which were fatal, resulting in a fatality rate of ~60% (World Health Organization: *http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_01_03/en/index.html*; accessed January 2008). Human infections mainly resulted from poultry-to-human transmission. Recently, however, there have been reports of human-to-human transmission,[4,5] increasing fears of a human pandemic.

H5N1 influenza is still a relatively novel disease with poorly understood pathology and pathogenesis. During the period from the first known outbreak nearly a decade ago until the present, only a limited number of reports describing pathological findings in human H5N1 cases has been published. Nevertheless, recent studies combined with early findings have gradually resulted in a better understanding of the cell and organ pathology caused by the H5N1 virus, as well as the viral tissue tropism. These findings together with animal and *in vitro* experiments have also contributed to a basic understanding of the pathogenesis of this disease. On the molecular level, several viral genes and gene products have been identified that may be responsible for the high pathogenicity of H5N1 influenza viruses. Herein, we describe the pathology of H5N1 avian influenza by reviewing the major pathological findings reported in hitherto published postmortem studies of human H5N1 cases as well as some key findings of animal studies. In addition, the major pathogenetic mechanisms and etiological factors of H5N1 influenza are discussed. The various molecular determinants of increased pathogenicity of H5N1 avian influenza viruses that have been identified in recent years are also presented. Finally, we have taken a closer look at the role of avian and human influenza virus receptors in relation to the transmissibility of the H5N1 virus.

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Address reprint requests to Jiang Gu, M.D., Ph.D., Department of Pathology, School of Basic Medical Sciences, Infectious Disease Center, Peking (Beijing) University, 38 Xueyuan Rd., Beijing, China 100083. E-mail: jianggu@bjmu.edu.cn.

Pathology

Histopathology

Thus far the results of only nine full autopsies including one autopsy of a fetus,[3,4,6–10] three limited autopsies (only the lungs and spleen),[10] and two cases of postmortem organ biopsies[11–13] have been reported. The main histopathological findings for each of these reports are summarized in Table 1 and discussed below.

The Respiratory Tract

The lungs typically show diffuse alveolar damage.[3,4,6–10] In cases with a short disease duration (<10 to 12 days), features of the exudative inflammatory phase of diffuse alveolar damage (edema, fibrous exudates, hyaline membranes) are predominant (Figure 1A).[3,7,9,11] In cases with a longer disease duration, changes consistent with the fibrous proliferative phase (organizing diffuse alveolar damage) and the final fibrotic stage (interstitial fibrosis) have been observed.[6–8,14] Hyperplasia of type II pneumocytes has been demonstrated in most autopsy cases.[3,6,8,11] Viral inclusions or other cytopathic changes have not been observed in pneumocytes.[6,8,11,14] Macrophages appeared to be the predominant cells within the alveoli,[3,7] whereas in the interstitium T lymphocytes, with or without neutrophils, are present.[3,6,7,11] Scattered histiocytes with hemophagocytic activity have been observed in the lungs of some cases.[6] The following additional histopathological features have also been reported: 1) desquamation of epithelial cells into alveolar spaces[4,8]; 2) bronchiolitis[8,9]; 3) cystically dilated air spaces[6]; 4) hemorrhage,[8,10,11] 5) pleuritis[9]; 6) features of interstitial pneumonitis,[4,7,8] and 7) apoptosis in alveolar epithelial cells and leukocytes.[9] Two cases of possible superinfections caused by fungi have been reported.[7,8] Because the above histopathological features are not unique to H5N1 influenza, it may be difficult to distinguish diffuse alveolar damage caused by H5N1 virus infections from diffuse alveolar damage caused by other microorganisms such as severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) or by other factors such as aspiration or oxygen toxicity. More specific tests such as *in situ* hybridization, reverse transcription-polymerase chain reaction (RT-PCR), and virus isolation are required to confirm H5N1 infection.

Other Organs

Reactive histiocytes with hemophagocytic activity have been noted in the spleen, lymph node, bone marrow, lungs, and liver,[3,4,6,9,11,13] although in recent autopsies the appearance of histiocytes has been less prominent or even absent.[7,8] Both hypercellular[3,13] and hypocellular[6] bone marrow have been reported. The spleen typically displays congestion and white pulp atrophy with depletion of lymphoid cells.[4,6,7,14] Lymph nodes show, apart from hemophagocytosis,[3,6] focal necrosis[6] and loss of germinal centers[7] in some cases. Apoptotic lymphocytes have been detected in both splenic and intestinal tissues.[9] Acute tubular necrosis has been found in several cases.[6,7,11,14] In liver tissue specimens, necrosis, activated Kupffer cells, cholestasis, and fatty changes have been observed.[6–9] In most instances the brain is edematous without any significant histopathological change,[6,7] whereas in two cases demyelinated areas and reactive histiocytes and foci of necrosis have been reported.[6,8] In other organs no remarkable histological changes have been observed.[3,7,8]

Among the H5N1 cases reported, there was a 24-year-old woman who was pregnant at the time of death. Both the placenta and the 4-month-old fetus have been studied.[7] The placenta showed foci of both syncytiocytotrophoblast necrosis and necrotizing deciduitis, and diffuse villitis. The fetal tissues did not display specific histopathological features, except for some edema and a few scattered interstitial neutrophils in the lungs.[7]

Virus Distribution

A number of studies have been performed applying immunohistochemistry (IHC) with monoclonal antibodies to hemagglutinin (HA) and nucleocapsid protein (NP) and/or *in situ* hybridization with sense and anti-sense probes to HA and NP to detect viral antigens and genomic sequences in various organs of H5N1 cases.[3,4,6–9] In addition, RT-PCR, strand-specific RT-PCR, and nucleic acid sequence-based amplification H5 detection assays have been performed to investigate virus tissue tropism.[3,6–9] According to early studies H5N1 infection appeared to be confined to the lungs.[3,6] However, the findings of recent studies indicate that the virus disseminates beyond the respiratory tract.[7–9] The main findings of these studies are summarized below.

The Respiratory Tract

Viral antigens and genomic sequences have been found in epithelial cells of the trachea[8] (Figure 1B) and alveoli (Figure 1C).[3,4,7–9] Tracheal epithelial cells were identified as both ciliated and nonciliated cells by double labeling with antibodies to tubulin.[7] The alveolar cells were identified as type II pneumocytes by double labeling with antibodies to surfactant protein (Figure 1D).[7,8] RT-PCR and nucleic acid sequence-based amplification-based H5 detection assays have detected viral RNA in both the trachea[7,9] and lungs.[7–9] Positive-stranded RNA has been detected in lung[7–9] and trachea[7,9] tissue samples. Because the H5N1 virus is a negative-stranded RNA virus, presence of positive-stranded RNA (mRNA and complement RNA, both necessary for viral replication) in a specific tissue suggests active viral replication at that site.

Brain

Viral sequences (Figure 1E) and antigens have been found in neurons of the brain[7] and H5N1 virus has been isolated from cerebrospinal fluid.[15] RT-PCR has detected

Table 1. Summary of Published Autopsy Reports and Their Main Histopathological Findings

Number of cases*	Disease duration/sex/age†	Main histopathological findings	Region/country/period/ reference (no.)
2 (FA)	29 days/F/13 years (case 1); 28 days/ F/25 years (case 2)	Lungs: DAD, interstitial fibrosis, reactive pneumocytes, interstitial lymphoplasmacytic infiltration, few histiocytes with reactive hemophagocytic activity, cystically dilated air spaces. Liver: central lobular necrosis. Kidneys: acute tubular necrosis. Brain: edema, demyelinated areas (not observed in case 2). Bone marrow: hypoplastic (case 1), hyperplastic (case 2), reactive histiocytes with reactive hemophagocytic activity. Lymph nodes: hemophagocytosis. Spleen: white pulp atrophy, reactive hemophagocytosis	Hong Kong/1997/(6)
1 (B)	11 days‡/M/3 years	Liver: microvesicular fatty changes (consistent with Reye's syndrome), multiple Councilman bodies with some inflammatory cells. Kidneys: vacuolation, vesicular changes in proximal tubules (consistent with Reye's syndrome). Bone marrow: occasional hemophagocytic activity, reactive changes	Hong Kong/1997/(12,13)
1 (B)	11 days/M/54 years	Lungs: reactive pneumocytes, hemorrhage, fibrinous exudates, sparse lymphocytic infiltration. Kidneys: acute tubular necrosis. Bone marrow: hypercellular, reactive hemophagocytosis	Hong Kong/1997/(11)
1 (FA)	6 days/M/33 years	Lungs: edema, hemorrhage, fibrin exudation, pneumocytes hyperplasia, intra-alveolar macrophages, interstitial T lymphocytes. Bronchial and hilar lymph nodes: reactive histiocytes with hemophagocytic activity. Bone marrow: hypercellular, hemophagocytosis. Spleen: lymphoid depletion. Other organs: no remarkable findings	Hong Kong/2003/(3,14)
1 (FA)	9 days/F/26 years	Lungs: DAD and interstitial pneumonia. Liver: cholestasis, hemophagocytic activity. Spleen: congestion, depletion of lymphocytes	Thailand/2004/(4)
1 (FA)	6 days/M/48 years	Lungs: DAD (exudative phase), atypical pneumocytes, bronchiolitis, pleuritis. Hemophagic activity in lungs, liver, and bone marrow	Thailand/¶/(9)
1 (FA)	17 days/M/6 years	Lungs: DAD (proliferative phase), interstitial pneumonia, focal hemorrhage, reactive pneumocytes, superimposed fungal infection, bronchiolitis. Lymph nodes, spleen, and bone marrow: slight histiocytic hyperplasia without hemophagocytic activity. Liver: mild fatty changes, activated Kupffer cells, lymphoid infiltration. Brain: edema, small foci of necrosis. Other organs: no remarkable findings	Thailand/2004/(8)
3 (FA)	9 days/F/24 years (case 1)§; 27 days/M/35 years (case 2)	Lungs: DAD, edema, intra-alveolar macrophages, desquamation of epithelial cells, foci with bronchopneumonia, areas with fibrosis (case 2). Spleen: massive depletion in white and red pulp. Lymph nodes: loss of germinal centers. Liver: edema, single cell hepatocyte necrosis. Kidneys: tubular necrosis. Brain (case 2): edema. Placenta (case 1): syncytiocytotrophoblast necrosis, diffuse villitis, necrotizing deciduitis. Other organs: no remarkable findings. Fetus: lungs: edema, features of mild interstitial pneumonitis. Liver: rare multinucleate giant cells. Other organs: no remarkable findings	China/2005/(7)
3 (LA)	NS‖	Lungs: DAD, reactive fibroblasts, hemorrhage. Spleen: atypical lymphocytes	Thailand/2004/(10)

*FA, full autopsy; LA, limited autopsy; B, biopsies.
†Disease duration before death in days (d); sex: female (F), male (M).
‡This patient died of H5N1 infection and the complications of Reye's syndrome.
§This patient was 4 months pregnant at the time of death.
¶Not specified.

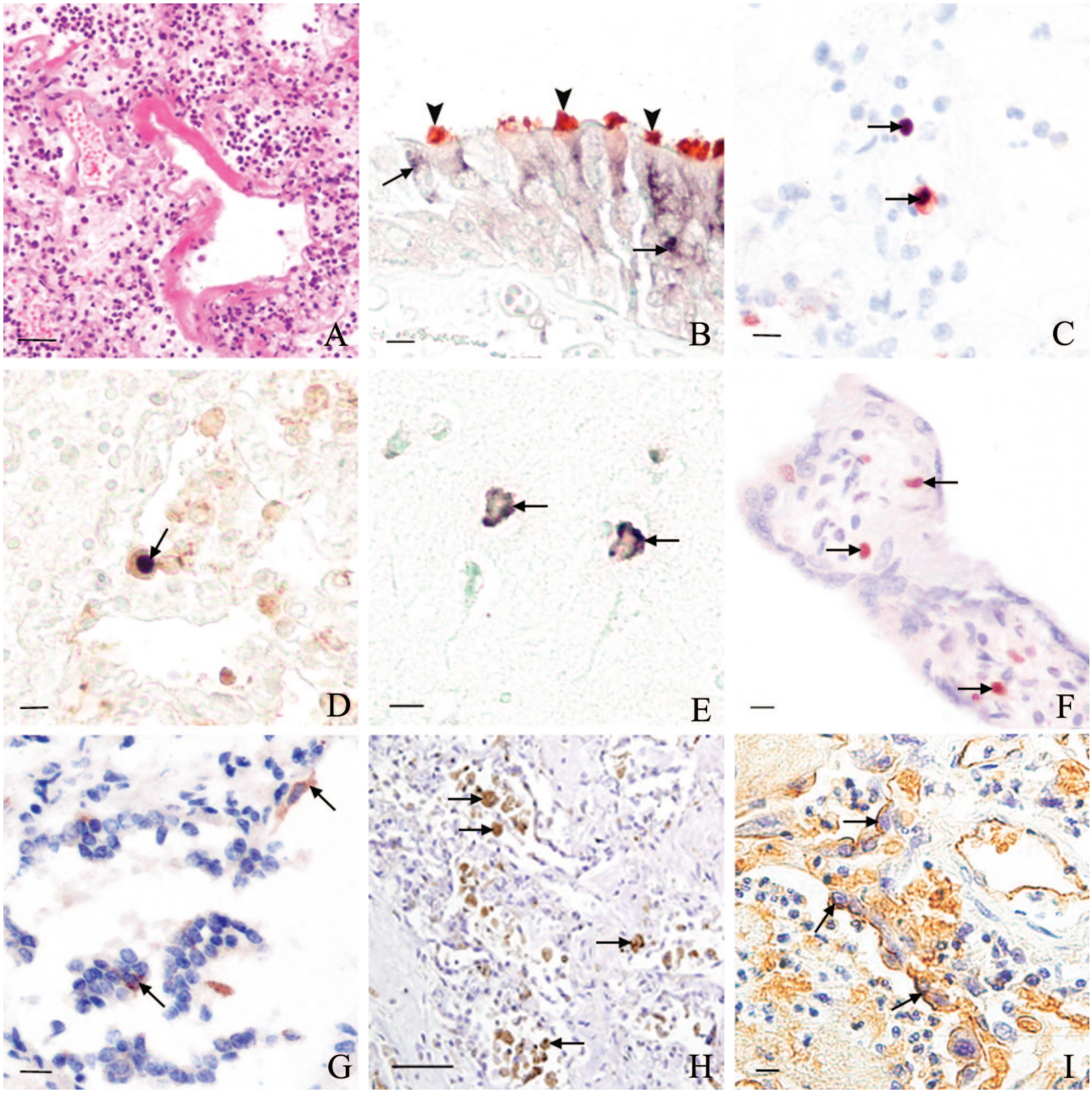

Figure 1. Examples of results of *in situ* hybridization, IHC, and lectin staining in various organs of H5N1 autopsies. **A:** Lung tissue showing severe damage, hyaline membrane formation, edema, fibrin exudation, and cellular infiltration (H&E staining). **B:** Double labeling with *in situ* hybridization (NP anti-sense probe) (purple-blue signals) and IHC with antibody to tubulin β (red signals, **arrowheads**) show positive *in situ* hybridization signals in the cytoplasm of both a tubulin-negative nonciliated cell (**arrow**) and a tubulin-positive (**asterisk**) ciliated cell in the trachea. **C:** Positive IHC staining (anti-NP antibody) in the nuclei and cytoplasm of some pneumocytes (**arrows**). **D:** Double labeling with *in situ* hybridization (NP sense probe) and IHC with antibodies to surfactant antibody A showing both dark blue nuclear *in situ* hybridization signals (**arrows**) and brownish-red cytoplasmic IHC signals (**arrowheads**) in a single cell in the lung. **E:** Positive *in situ* hybridization signals (NP sense probe) in the cytoplasm of some cells (**arrows**) in brain tissue taken from the parietal lobe. Double labeling with antibodies to neuron-specific enolase identifies these cells as neurons (not shown). **F:** Positive IHC signals (anti-NP antibody) in large mononuclear cells (**arrows**) with morphological features of macrophages within the core of a chorionic villus (**arrows**). IHC with antibody to CD68 on consecutive sections shows that these cells are most likely Hofbauer cells (fetal macrophages) (not shown). **G:** Positive IHC signals (anti-HA antibody) in the cytoplasm of some pneumocytes in fetal lung tissue. **H:** IHC with antibodies to macrophage inflammatory protein-1α shows a large number of positive cells in lung tissue. **I:** Staining with *Maackia amurensis* lectin II (specific for α-2,3-linked sialic acids) detects the presence of avian influenza virus receptors on pneumocytes. **A**, **C**, **D**, and **F** involve tissues taken from a 24-year-old pregnant female infected with H5N1 virus who died 9 days after disease onset. **B**, **E**, **H**, and **I** are taken from a 35-year-old male H5N1 patient who died 27 days after disease onset. **G** is lung tissue of the fetus carried by the 24-year-old pregnant female. **B**, **D**, and **E:** The *in situ* hybridization probes were labeled with digoxigenin and a NBT/BCIP substrate chromogen kit (Promega Corp., Madison, WI) was used to visualize the *in situ* hybridization signals, resulting in a purplish blue color. Anti-HA and anti-NP antibodies were purchased from Beijing Perfect Biotechnology Ltd. (Beijing, China) and VivoStat Inc (Portland, ME), respectively. **B–D** and **F:** The IHC reaction products were detected with 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO), which gives a brownish-red color. **G–I:** The IHC reaction products were colorized with diaminobenzidine (Zymed Laboratories, South San Francisco, CA), which gives a brown reaction color. **C** and **F–I** are counterstained with hematoxylin. **B** and **E** are lightly counterstained with methyl green. Scale bars: 25 μm (**A**, **C**, **D**, **F**, **G**); 10 μm (**B**); 12.5 μm (**E**, **I**); 20 μm (**H**).

both negative- and positive-stranded RNA in the brain. Dissemination to the central nervous system may be blood-borne or may alternatively occur via the afferent fibers of the olfactory, vagal, trigeminal, and sympathetic nerves after replication in the lungs, as has been observed in a mouse model.[16]

Intestines

Viral genomic sequences have been detected in epithelial cells of the intestines by *in situ* hybridization.[7] RT-PCR has detected both negative- and positive-stranded RNA in the intestines.[7,8] These findings are consistent with reports of viral shedding in stool samples, as detected by RT-PCR and viral isolation,[15,17] and with frequently observed clinical symptoms related to the gastrointestinal tract.[2,18,19] Because avian influenza viruses maintain sialidase activities, despite the low pH conditions in the upper gastrointestinal tract, infection of the intestines may be the result of ingestion of infected secretions.[20] In contrast to viral sequences, viral antigens have not been detected in the intestines.[7,8] The reason for this is at present undetermined.

Other Organs

In situ hybridization and IHC are both negative for the heart, spleen, kidneys, and liver. In contrast, RT-PCR and nucleic acid sequence-based amplification-based H5 detection assay are positive for these organs.[7] The discrepancies between the *in situ* hybridization/IHC and RT-PCR results may be explained by either false-negative results of the *in situ* hybridization and IHC assays attributable to limitations in sensitivity or false-positive RT-PCR results attributable to viremia in blood perfusing the organs without actual viral replication in the tissues.[21]

Placenta and Fetus

In the placenta of a female infected with H5N1 influenza virus, viral antigens and sequences have been found in Hofbauer cells (fetal macrophages) (Figure 1F) and cytotrophoblasts, but not in syncytiotrophoblasts.[7] RT-PCR results have indicated viral replication in the placenta.[7] In addition, *in situ* hybridization, IHC (Figure 1G), and real-time RT-PCR confirmed infection of the fetus, demonstrating that the virus is vertically transmissible from mother to fetus.[7] Transplacental transmission of the virus may occur through mechanisms similar to those for transmission of the cytomegalovirus, a virus that is also known to infect mainly cytotrophoblasts and Hofbauer cells.[22] Transmission may take place via transcytosis across syncytiotrophoblasts to cytotrophoblasts in chorionic villi.[22] Alternatively, the virus may infect invasive cytotrophoblasts within the uterine wall after contact with maternal blood. These infected cells would subsequently transmit the virus via the cell columns to the anchoring chorionic villi.[22] The virus may then be transmitted to Hofbauer cells, which would enter the fetal circulation and carry the virus to the fetus.

Immune Cells and Blood

Viral sequences and antigens have been detected in lymphocytes in lymph node tissue, as well as in Hofbauer cells (macrophages of the placenta), Kupffer cells (macrophages of the liver), and mononuclear cells in the intestinal mucosa.[7] These findings are consistent with *in vitro* experiments demonstrating infection of macrophages by the H5N1 influenza virus[23–25] and with *ex vivo* experiments showing H5N1 virus attachment to and infection of alveolar macrophages in human lung tissue.[26,27] Viral RNA has been detected in blood samples of several H5N1 cases, all of them fatal.[17] Viremia may occur in the course of the disease, as evidenced by virus isolation from serum and plasma samples of two fatal cases.[15,28] Accordingly, extra-pulmonary dissemination may be the result of viremia or of infected immune cells transporting the virus to other organs.

Histopathology and Virus Distribution in Animal Studies

The available studies of human H5N1 autopsies have a number of limitations in terms of pathological findings. The majority of the individuals who died from H5N1 influenza had received various interventional therapies aimed at limiting tissue injury and viral replication. Second, none of these cases succumbed during the early phase of the infection (see Table 1), thus preventing histopathological and molecular pathological data from being obtained at the very early phase of the illness through autopsy. Animal studies have provided important supplementary information with respect to the natural course of H5N1 influenza. Several animal models including the mouse, ferret, cynolmolgus macaque, and cat have been used to investigate viral replication and histopathology in H5N1 infections. Histopathologically, early lesions in the lungs included features of focal peribronchiolar pneumonia (3 to 5 days after infection), whereas 6 to 8 days after infection the lungs showed extensive consolidation and bronchiolitis.[29–39] In the majority of these animal models, tissue injury is also observed to varying degrees in extrapulmonary organs, in particular in the brain.[29–33,35,36] Similar to human cases, the virus appears to be capable of spreading beyond the lungs as has been evidenced by virus isolation and detection of viral antigens in various extra-pulmonary organs including the brain, liver, lymphoid tissues, heart, and kidneys.[29–35,37]

Infection with highly pathogenic H5N1 isolates in animal experiments has been associated with severe lymphopenia. In these experiments, H5N1 virus caused progressive depletion of lymphocytes, whereas infection with low pathogenic virus did not affect total white blood cell counts in mice.[38,39] In human cases, lymphopenia has been associated with disease severity,[2] and lower numbers of T lymphocytes have been detected in fatal cases compared to nonfatal cases.[17] Several mechanisms have been implicated in the genesis of lymphopenia, including apoptosis and bone marrow suppression.

Implications on Pathology

H5N1 influenza appears to be a systemic infection in both human and animal cases. In humans the trachea, brain, and intestines may be infected in addition to the lungs. It appears that the virus may also spread to other organs, such as the kidneys and liver, as has also been demonstrated in animals. To gain a better understanding of viral distribution in humans, additional autopsy studies with molecular methods will be necessary. In view of adequate treatment of patients with avian influenza, it is important to realize that the disease affects multiple organs. Therapeutic regimes should therefore not only comprise optimal respiratory care but should also pay attention to adequate supportive care of other organs involved. Multiple-organ infection and vertical transmission of H5N1 have public health implications. The fact that the virus has been isolated from serum and feces makes it possible that infection can be transmitted through gastrointestinal contamination or infected blood. Needless to say that extreme care should be taken when handling body fluids from H5N1 cases. The finding that the virus is transmissible from mother to fetus is alarming and might reflect enhanced pathogenicity of the H5N1 virus. Care should be taken when handling delivery or abortion from H5N1-infected mothers.

Pathogenesis

Various factors are thought to be involved in the pathogenesis of H5N1 influenza (Figure 2), and a combination of these factors most likely determines the extent of tissue injury and disease outcome. The role of dysregulation of cytokines and chemokines has been studied extensively and may be one of the key mechanisms in the pathogenesis of H5N1 influenza, in addition to injury resulting from viral replication. Other factors, such as up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and reduced cytotoxicity of $CD8^+$ lymphocytes are also believed to be involved in the pathogenesis, although their exact roles are less clear at present. We discuss these factors and related mechanisms below.

Viral Replication

It is generally thought that replication of the H5N1 virus results in cell and organ damage by either cytolytic or apoptotic mechanisms, similar to human influenza infections. There are clear indications of active viral replication in the respiratory tract. The virus has been isolated from throat and trachea aspirates, and postmortem lung tissues.[3,17,18,40] Viral RNA has been detected in nasal, nasopharyngeal, and tracheal specimens.[17,18,40,41] Viral RNA has been detected in nasopharyngeal aspirates ranging from 1 day up to 15 days after disease onset.[40,42] Viral replication appears to be prolonged in H5N1 influenza because viral loads when plotted against time did not show a clear decline in a large group of H5N1 patients.[17] In the same group of H5N1 cases, viral RNA levels in pharyngeal and nasal specimens were higher than in a group of patients infected with common human influenza viruses. In addition, the highest viral loads were

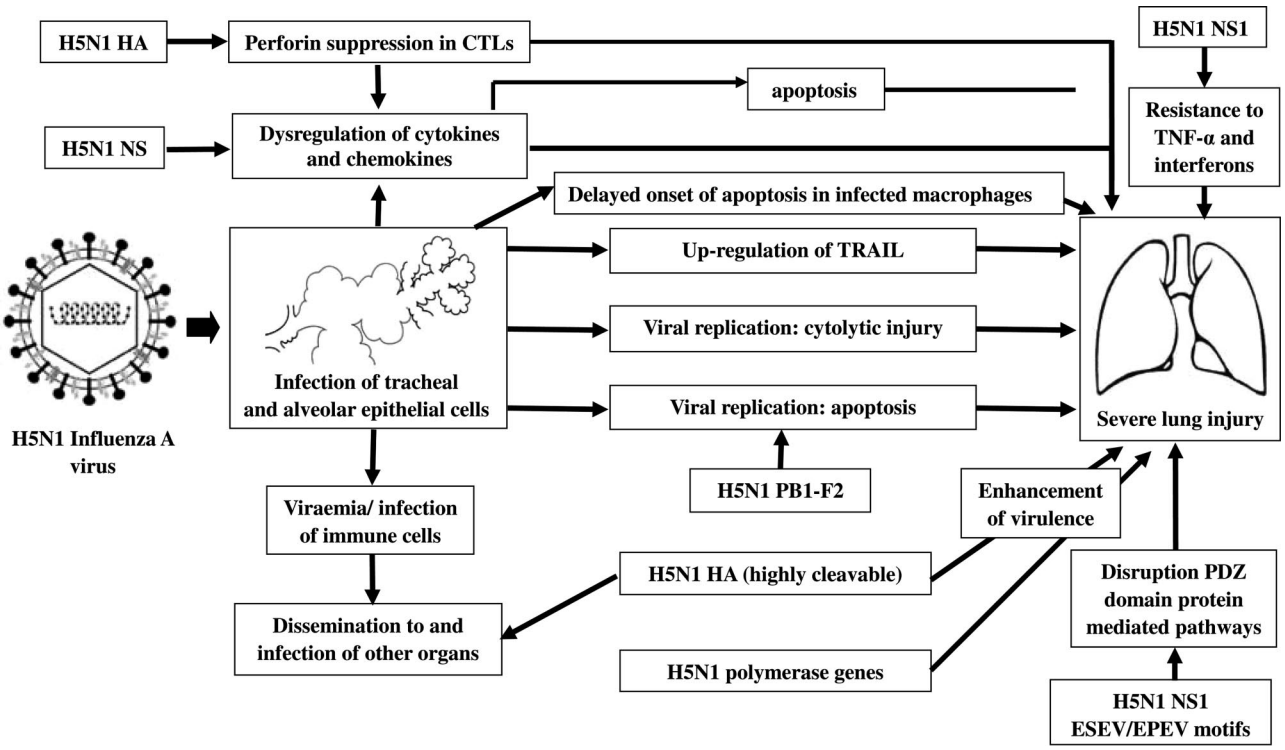

Figure 2. Proposed pathogenesis of human H5N1 infection. Diagram depicting the key pathogenetic mechanisms, viral genes, and gene products that may be involved in H5N1 influenza virus infection. CTLs, cytotoxic T lymphocytes.

detected in the fatal cases, suggesting a correlation between viral replication and negative disease outcome.[17] Both *in situ* hybridization with anti-sense probes and RT-PCR detecting positive-stranded RNA have provided evidence of viral replication in the trachea and lungs (see Virus Distribution).[7] In addition, *ex vivo* experiments have shown that H5N1 viruses can productively infect tissues of the nasopharynx and lungs.[27] In animal models the H5N1 virus has also been detected in the upper respiratory tract and the lungs as early as 1 day after infection, replicating to extremely high levels.[32,34,36,37,39] In contrast to the respiratory tract, little has been reported regarding H5N1 viral replication in extra-pulmonary organs. Positive-stranded RNA has been detected in the intestines, brain, heart, and placenta (see Virus Distribution). In addition, anti-sense probes have been found to hybridize in the intestines, brain, and placenta.[7] These findings strongly suggest that active viral replication may occur in these organs. This would be consistent with findings in animal experiments in which high replication titers of human H5N1 isolates have been found not only in the respiratory tract, but also in several extra-pulmonary organs (see Histopathology and Virus Distribution in Animal Studies).[29–35,37,39] In these studies viral replication peaked in the extra-pulmonary organs on days 3 to 6 after infection.[31,32,37,39]

Dysregulation of Cytokines and Chemokines

Various studies indicate that aberrant production of proinflammatory cytokines and chemokines may play an important role in the pathogenesis of H5N1 influenza. Pathological features that are consistent with dysregulation of cytokines and chemokines, including hemophagocytotic activity, have been described in H5N1 autopsy cases (see Histopathology).[3,4,6,9,11,13] In many H5N1 patients elevated serum levels of proinflammatory cytokines and chemokines have been detected.[3,6,8,17] In a study cohort of 18 H5N1 patients, serum levels of most of the tested chemokines and cytokines were significantly higher than in the control group of H3N1 human influenza patients.[17] In the same study group serum cytokine and chemokine levels have been found to correlate with viral loads in pharyngeal specimens, suggesting that high viral loads may induce hypercytokinemia and hyperchemokinemia.[17] Suppression of viral replication by timely administration of antiviral agents may therefore help prevent hyperinduction of cytokines and chemokines.

Serum cytokine and chemokine levels do not necessarily reflect local production of these regulatory proteins in the lungs.[43] There are a number of studies investigating the local expression of cytokines and chemokines in the lungs of H5N1 cases. Immunohistochemistry has detected high expression of tumor necrosis factor-α (TNF-α) in the lungs of a H5N1 autopsy case in Hong Kong.[3] In another investigation the lungs of a H5N1-infected case showed enhanced expressions of macrophage inflammatory protein-1α, regulated on activation normal T cell expressed and secreted (RANTES), interferon-γ, interferon-β, and interleukin-6, but neither of monocyte chemoattractant protein 1 (MCP-1) nor of TNF-α (R.S. Deng, unpublished observations) (Figure 1H). Furthermore, up-regulation of TNF-α has been detected in two autopsy cases by using RT-PCR.[8,9] It should be noted that the availability of data regarding serum cytokine levels and immunohistopathological studies in humans is limited. In addition, interpretation of cytokines and chemokines in critically ill patients is not without difficulties. In view of these difficulties, *in vitro* and animal studies could provide additional information. Results of *in vitro* experiments support the role of an exaggerated immune response in the pathogenesis of H5N1 influenza. H5N1 avian influenza viruses induce significantly higher expression of several cytokines and chemokines in human macrophages and respiratory epithelial cells than human influenza viruses.[23,24,44,45] In these experiments enhanced expression is reflected by increased production of cytokines and chemokines in the supernatants from infected cells.[23,24,45] Animal experiments have also provided support for a possibly critical role of proinflammatory cytokines and chemokines in H5N1 pathology (see Nonstructural Proteins).[46–48] Up-regulation of cytokines and chemokines, however, is not a unique feature of H5N1 influenza infection. In SARS, hyperinduction of the immune system is believed to be an important pathogenetic factor.[49,50] Similarly, up-regulation of cytokines and chemokines is also a significant characteristic of the H1N1 human influenza virus that caused a major influenza pandemic in 1918 to 1919.[51–53] High expression of cytokine and chemokine genes have been found in the lungs of mice and nonhuman primates infected with the reconstructed 1918 H1N1 influenza virus.[52,53] H5N1 viruses may not only be capable of up-regulating cytokines and chemokines, but also be resistant to the anti-viral effects of interferons and TNF-α (see below).

Up-Regulation of TRAIL and Apoptosis

Up-regulation of functional TRAIL in macrophages infected with the H5N1 virus may be another important factor in the pathogenesis of H5N1 influenza virus infection.[54] TRAIL is one of the many death receptor ligands that trigger apoptosis of cells by binding to death receptor ligand receptors expressed on target cells. Zhou and colleagues[54] have demonstrated significantly higher expression of TNF-α and TRAIL in macrophages infected with H5N1 virus *in vitro* than in macrophages infected with human H1N1 influenza virus. In addition, T lymphocytes co-cultured with macrophages infected with H5N1 virus show increased induction of apoptosis compared to T lymphocytes co-cultured with macrophages infected with other influenza viruses. Enhanced sensitization of virus-infected T lymphocytes to death receptor ligand-induced apoptosis has also been demonstrated.[54] Both sensitization and up-regulation of TRAIL may partially account for the lymphopenia and lung injury frequently observed in H5N1 patients.[54] In addition, a delayed onset of apoptosis has been demonstrated *in vitro* in H5N1-infected macrophages compared with H1N1-infected macrophages.[25] Prolonged survival of infected macrophages may further

enhance the induction of apoptosis in T lymphocytes.[25] It may also augment immune-mediated pathology because macrophages secrete cytokines and chemokines for a longer period of time. In human autopsies apoptosis has been detected in both alveolar epithelial cells and leukocytes in the lungs, as well as in spleen and intestinal tissues.[9] Apoptosis may, therefore, be one of the pathogenetic mechanisms contributing to injury in the lungs and other organs. Apoptosis may occur as a result of direct viral replication or up-regulation of cytokines and chemokines.[9]

Reduced Cytotoxicity of $CD8^+$ Lymphocytes

As opposed to H1N1 and H3N2 viruses, the HAs of H5N1 viruses suppress perforin expression in cytotoxic T lymphocytes according to *in vitro* experiments.[55] It has been suggested that this may result in impaired cytotoxic activity causing failure of clearance of H5N1 virus or HA (H5) protein-bearing cells, including antigen-presenting cells. Excessive production of interferon-γ caused by sustained antigenic stimulation of cytotoxic T lymphocytes may subsequently lead to up-regulation of proinflammatory cytokines in macrophages.[55]

Implications on Pathogenesis

H5N1 viral replication appears to be prolonged with high levels of viral RNA, and the virus may disseminate to extra-pulmonary organs. Timely suppression of viral replication is the mainstay of therapy in H5N1 infection. Oseltamivir, a neuraminidase inhibitor is the principal antiviral agent of choice because many H5N1 isolates are resistant to amantadines (World Health Organization: Clinical management of human infection with avian influenza A (H5N1) virus. *http://www.who.int/csr/disease/avian_influenza/guidelines/clinicalmanagement07.pdf*; accessed January 2008). Given the potential role of up-regulation of cytokines and chemokines in the pathogenesis of H5N1 infection, it has been suggested that suppression of the exaggerated host immune response may also be a beneficial therapeutic strategy. Unfortunately, the data on treatment with corticosteroids, albeit limited, have thus far not shown any obvious clinical benefit in treatment of human H5N1 infection.[41] Similarly, corticosteroids have failed to show any clear benefits in the treatment of other viral respiratory infections, including SARS.[56] Further, no human trial with specific cytokine blockers for treatment of H5N1 influenza has been published to date. As to the use of cytokine blockers in animal experiments, only one study has been published describing reduced illness severity in human influenza virus-infected mice treated with anti-TNF antibodies.[57] Altogether, there is insufficient data supporting the use of immunomodulating agents in the treatment of H5N1 influenza. There is an obvious need for more studies into pathogenetic factors as well as therapeutic intervention strategies including immunotherapy.

Viral Genes and Gene Products Involved in the Pathogenesis of H5N1 Influenza

Influenza viruses belong to the family of *Orthomyxoviridae*. There are three types of influenza viruses: type A, type B, and type C. Avian influenza viruses are all classified as type A influenza viruses.[58] Influenza viruses can be subtyped based on the antigenicity of their two surface glycoproteins, HA and neuraminidase (NA).[58,59] Sixteen HA and nine NA subtypes have thus far been identified.[59,60] The influenza A virus genome consists of eight gene segments encoding 11 viral proteins (gene products) including HA, NA, polymerase proteins (PB1, PB2, PA, and PB1-F2), NP, nonstructural proteins (NS1 and NS2), and M1 and M2 proteins (Figure 2).[61,62] These genes and/or gene products have various basic functions ranging from viral RNA synthesis to receptor binding (Table 2).[58,59] Several of these genes and/or gene products have additional functions that may contribute to the pathogenesis of H5N1 influenza and enhance pathogenicity of H5N1 influenza viruses. Both the basic functions of influenza A viral proteins and the specific functions accounting for increased pathogenicity are summarized in Table 2 and discussed in the following sections. Figure 2 contains a schematic presentation of the viral genes and gene products that may be involved in the key pathogenetic mechanisms of H5N1 influenza.

Hemagglutinin

Hemagglutinin is a surface protein that functions as a receptor-binding site and is the target of infectivity-neutralizing antibodies.[63] The HA protein attaches to sialic acid-containing receptors expressed on the host cell, and after proteolytic activation of the precursor HA molecule into HA1 and HA2, the virus fuses with the host cell.[58] Early reverse genetics studies have demonstrated that the level of cleavability of HA determines the virulence of avian influenza viruses in poultry.[64] Avirulent viruses usually possess HAs with a single arginine residue at the cleavage site that can only be cleaved by extracellular trypsin-like proteases present in the upper respiratory and gastrointestinal tracts, thus merely giving rise to local infections. In contrast, virulent viruses have HAs with multiple residues at the cleavage site that can be activated by ubiquitous intracellular proteases and may therefore cause systemic infections.[65] The significance of multiple residues at the cleavage site has also been established for the virulence of H5N1 viruses, as evidenced by attenuation of disease in mice infected with a mutant H5N1 A/Hk/483/97 virus with a single arginine residue at the cleavage site.[66] All H5N1 viruses isolated from human cases since 1997 have multiple basic amino acids at the cleavage site.[1,12,45,66–72] However, the disease severity in human cases varies from mild to extremely severe, implying that there are other factors responsible for the virulence of H5N1 influenza viruses in humans.[38]

The destructive 1918 influenza virus resembles the H5N1 virus in having a HA that is highly cleavable. The

Table 2. Main Basic Functions of Influenza A Viral Proteins and H5N1 Viral Proteins Most Likely Contributing to Pathogenicity

RNA segment	Viral gene product	Basic functions	H5N1 viral proteins contributing to pathogenicity
4	HA	Receptor binding site, membrane fusion, main target for neutralizing antibodies	Multiple residues at cleavage site increase virulence (64,66), H5 suppresses perforin expression in cytotoxic T cells (Vn/1203/04)*(54)
6	NA	Cleavage of progeny virions from host cell receptors, minor target for neutralizing antibodies	Histidine to tyrosine substitution at position 274 confers resistance to oseltamivir (78,79)
8	NS	NS1 participates in processing of mRNA, NS1 antagonizes host innate and adaptive immune response, NS2 controls export of RNP from nucleus	Glutamic acid at position 92 of NS1 confers resistance to TNF-α and interferons (Hk/156/97, Hk/483/97, Hk/486/97)* (88), Glu-Pro-Glu-Val (EPEV) and Glu-Ser-Glu-Val (ESEV) motifs at the C-terminus of NS1 may disrupt important cell signaling pathways (85), NS gene contributes to dysregulation of cytokines and chemokines (Hk/156/97)* (24,47)
7	M1	Virus assembly, major component of virion	
	M2	H^+ channel controls pH during virus uncoating and HA synthesis	Serine to asparagine substitution at position 31 confers resistance to amantadine (67,69)
1, 2, 3	PB1, PB2, PA, PB1-F2, NP	The polymerases (PB1, PB2, and PA) and NP form the ribonucleoprotein complex that plays a role in RNA replication and transcription. PB1-F2 induces apoptosis	Lysine at position 627 of PB2 enhances pathogenicity and promotes replication in cells of the upper respiratory tract, at lower temperatures (Hk/483/97, Vn1203/04)* (66,83). The PB1-F2 gene is under strong positive selection pressure in avian influenza isolates (85). Serine at position 66 of PB2-F1 increases virulence (various highly virulent Hk/97 viruses)* (46)

*In parentheses are the particular H5N1 isolates for which the characteristic has been demonstrated.

HAs of both virus types can be cleaved in the absence of trypsin.[65,73] However, the mechanisms for the high cleavability of HA differ for both virus types. In contrast to the H5N1 viruses, of which the HA is easily cleavable because of the presence of multiple basic amino acids at the cleavage site, both HA and NA appear to be involved in the HA activation of the 1918 H1N1 viruses through a yet unidentified mechanism.[73] As discussed in Reduced Cytotoxicity of $CD8^+$ Lymphocytes, the HA of H5N1 viruses may also be involved in the suppression of perforin in cytotoxic T lymphocytes.

Neuraminidase

The NA protein is a sialidase that cleaves the HA of progeny virions from the sialic acid-containing receptors on the surface of the host cells, thus separating the particles from the infected cells in which they were generated.[59] The H5N1 viruses isolated during the 1997 outbreak have a deletion of 19 amino acids in the NA stalk region[1,12,72,74] that is thought to play a role in the adaptation of the virus in the course of transmission from aquatic to terrestrial birds.[74] The first 2003 human isolates had no NA stalk deletion,[47,71] but more recent human and chicken isolates did show a deletion in the NA stalk, which is similar, although not identical, to that of 1997 H5N1 viruses.[67,68,70,75] Viruses containing an NA stalk deletion are less capable of freeing virions from infected cells.[74] This negative effect may, however, be counterbalanced by the facilitating effects on the release of viral particles conferred by an additional glycosylation site at the top of HA globular heads,[74,76] frequently found in H5N1 viruses with an NA stalk deletion.[67,70]

Most H5N1 viruses are sensitive to neuraminidase inhibitors.[67,77] It is remarkable that in three human H5N1 cases a histidine to tyrosine substitution at position 274 of the NA protein that confers resistance to oseltamivir (a neuraminidase inhibitor), has been reported.[78,79] All instances were related to prophylactic or therapeutic use of oseltamivir and may have been caused by incomplete suppression of viral replication.[78]

Polymerase Gene Complex and Nucleocapsid Protein

The polymerase complex is composed of three viral polymerase proteins (PB1, PB2, and PA) involved in viral RNA synthesis.[59] The polymerase complex together with NP constitutes the ribonucleoprotein complex.[58] The RNA gene segments are encapsulated by NP, facilitating their recognition by the polymerase complex.[58] The polymerase gene complex is an important molecular determinant of virulence in animal models.[66,80,81] Early studies have demonstrated that the amino acid at position 627 of PB2 determines virulence of H5N1/Hk/97 human isolates in mice. Glutamic acid at this position confers low pathogenicity, whereas lysine at this position confers high pathogenicity.[66] Glutamic acid to lysine substitutions have also been detected in several viruses isolated from human cases in Vietnam and Thailand.[68–70,82,83] H5N1/Vietnam/2004 virus isolates possessing lysine at position 627 have

been shown to replicate better in a wider range of cell types including cells of the upper respiratory tract and at lower temperatures than similar isolates with glutamic acid at this position, thus possibly facilitating virus excretion by sneezing and coughing.[83] It is noteworthy, however, that the presence or absence of lysine at position 627 does not appear to affect the clinical outcome in humans.[17,69] Moreover, several viruses have been isolated from fatal human cases that lack this substitution.[17,45,70,71,82] This would indicate that lysine at position 627 is not a prerequisite for high virulence in humans.

Studies in mice and ferrets infected with reassortant viruses containing genes of A/Vietnam/1203/04 have demonstrated that polymerase complex genes, rather than *HA* or *NA* genes, account for the high virulence of this particular H5N1 strain.[80] In this study, not only PB2 but also PB1 contributes to pathogenicity, as suggested by attenuated disease in mice inoculated with PB1 reassortants.[80] To explain the molecular basis of adaptation of influenza viruses to a new host species, a model of species transmission has been designed using a low pathogenic avian influenza virus and its lethal mouse adapted descendant.[81] In this model various mutations in the ribonucleoprotein complex were found to enhance pathogenicity.[81] Remarkably, mutations similar to the ones detected in this mouse model have also been detected in mammalian and human strains that had only shortly been transmitted from poultry.[17,81] This may indicate that the polymerase complex plays an important role in the adaptation to a new host.[80,81] Such a role is further supported by findings regarding the 1918 influenza virus. Similar to the H5N1 virus, the 1918 influenza virus appears to be an avian-like virus rather than a reassortant.[84] Only 10 amino acid changes have been found to differentiate the polymerase proteins of the 1918 human influenza virus from avian consensus sequences.[84] It is thought that these changes were essential for the adaptation of the 1918 influenza virus to humans.[84] Several similar changes, including the lysine residue at position 627, have also been independently detected in H5N1 viruses isolated from human cases.

In a large-scale study of avian influenza isolates, the gene encoding for PB1-F2 was found to be the only gene under positive selection.[85] PB1-F2 is a small mitochondrial protein that is encoded on an open reading frame of PB1.[61] This open reading frame is highly conserved in avian influenza isolates.[85] PB1-F2 sensitizes infected cells to apoptotic stimuli such as TNF-α through the interaction with the mitochondrial permeability transition pore complex.[86] Increased cell death responses in mice infected with the reconstructed 1918 influenza virus have been linked to the PB1-F2 protein.[52] It has been speculated that the PB1-F2 protein of the H5N1 virus has a similar role in the pathology in H5N1 infections. In addition, it may be possible that PB1-F2 also induces apoptosis of immune cells, which could result in diminished antigen presentation leading to an insufficient adaptive immune response.[46]

Recent recombinant virus studies have demonstrated that a single mutation in the PB1-F2 protein [serine (S) instead of asparagine (N) at position 66] of H5N1 (Hk/97) increases viral pathogenicity. Mice infected with this virus showed decreased survival rates, significantly higher viral loads in the lungs, delayed viral clearance, as well as elevated levels of cytokines in the lungs.[46] Slower viral clearance, induced by the expression of the PB1-F2 protein, may cause immune-mediated injury, supported by the detection of increased levels of cytokines in the lungs.[46]

Nonstructural Proteins

The NS proteins (NS1 and NS2) are viral proteins that play a significant role in viral replication.[58] The NS1 protein is crucial for evading the innate immune response of the host by inhibiting the antiviral response mediated by type I interferons.[87] The *NS* gene of certain H5N1 viruses may play an additional role in the pathogenesis of H5N1 influenza because it may confer resistance to the antiviral effects of TNF-α and interferons. *In vitro* experiments have shown that the replication of H5N1/97 viruses in porcine lung epithelial cells was not inhibited by pretreatment with either interferons or TNF-α.[88] The presence of glutamic acid at position 92 of NS1 appeared to be a prerequisite for the resistance to antiviral cytokines.[88] This *in vitro* resistance is supported by results of *in vivo* animal experiments in which pigs infected with a reassortant influenza virus (H1N1) bearing the NS gene of the H5N1/97 virus display a more severe disease compared to pigs inoculated with the parental H1N1 virus.[72] Recent human and avian isolates, however, lack glutamic acid at position 92 of the NS1 protein.[17,45,68,82]

The *NS* gene of H5N1 viruses may also account for up-regulation of cytokines and chemokines. High concentrations of proinflammatory cytokines and low concentrations of an anti-inflammatory cytokine (interleukin-10) have been detected in lung homogenates of mice infected with a reassortant influenza virus encoding the *NS* gene of the H5N1/97 virus. Similar cytokine imbalances have not been found in mice inoculated with a reassortant influenza virus encoding the *NS* gene of the low pathogenic H5N1/2001 virus or an *NS* gene encoding a glutamic acid to asparagine substitution at position 92 of NS1.[47] Furthermore, TNF-α concentrations in the supernatants from macrophages infected with recombinant viruses encoding the *NS* gene of the H5N1/97 are significantly higher than in those from macrophages infected with recombinant viruses containing the *NS* gene of nonrelated influenza viruses.[24] It has been argued that both increased resistance to antiviral effects of cytokines and up-regulation of cytokine production may act synergistically to induce pulmonary injury.[24]

Two PDZ ligand (PL) sequence motifs in the *NS1* gene of H5N1 viruses have recently been identified as potential co-determinants of virulence. Viruses isolated during the 1997 outbreak contain a Glu-Pro-Glu-Val (EPEV) motif at the carboxyl terminus of NS1, and 2003 to 2004 isolates contain a Glu-Ser-Glu-Val (ESEV) motif. These avian PLs bind *in vitro* to the PDZ domains of several human proteins that are crucial for cell signaling.[85] Infection of human cells by viruses with avian PL motifs may therefore

disrupt several PDZ-domain protein-mediated pathways, thus contributing to pathogenicity of H5N1 viruses.[85] However, recently isolated, highly pathogenic viruses lack these motifs. The presence of EPEV or ESEV motifs at the carboxyl terminals of NS1 proteins thus appears not to be a prerequisite for virulence of H5N1 viruses.[89]

M1 and M2 Proteins

The *M* gene encodes two proteins: M1 (matrix protein) and M2. The matrix protein lies underneath the viral envelope and plays a significant role in virus assembly.[65] M2 is a small protein embedded in the viral envelope that functions as a H^+ ion channel, thus controlling the pH in the Golgi complex during HA synthesis and virion disassembly.[62] In a study of Thai and Indonesian isolates, the gene encoding the M2 protein was found to be one of the two gene segments (in addition to PB1-F2) under positive selection, indicating a possible role for M2 in the adaptation of the virus to a new host.[69] Most viruses isolated from humans and/or birds in countries on the Indo-China peninsula (the so-called "clade 1 viruses") contain a serine to asparagine substitution at residue 31 of the M2 protein, which is associated with resistance to amantadines.[67,69] In contrast, only few viruses isolated from humans and/or birds in China, Indonesia, Japan, and South Korea (the so-called "clade 2 viruses") possess such a substitution.[7,77,80,90]

Implications for Viral Genes and Gene Products

Since the first avian influenza outbreak in 1997 several amino acid substitutions including the glutamic acid to lysine substitution at position 627 of PB2 and glutamic acid at position 92 of NS1 have been indicated as major contributors to virulence. In view of the observations that recent H5N1 viruses, including the ones isolated from fatal cases, lack these substitutions and that recent isolates have been found to posses other mutations, it becomes increasingly apparent that virulence cannot be attributed to a single gene or amino acid substitution. In fact virulence appears to be a polygenic trait with several genes co-operating together. The precise role of the recently discovered PB1-F2 protein in the pathogenesis of H5N1 influenza requires further exploration. Future studies will most likely identify other molecular determinants of pathogenicity.

Receptor Specificity and Transmissibility of the H5N1 Virus

Avian Versus Human Influenza Virus Receptors

Human and avian viruses bind to different receptors. The HA protein of avian influenza viruses preferentially binds to sialic acids linked to galactose by α-2,3 linkage (avian influenza virus receptors), which are located on the intestinal epithelial cells of avians. In contrast, the HA protein of human influenza viruses primarily recognizes α-2,6-linked sialic acids (human influenza virus receptors), which are notably expressed on epithelial cells of the human trachea.[91–95] Because of these differences in receptor specificity and distribution, as well as the limited replication in humans, avian influenza viruses were initially thought to be incapable of causing human infection. However, this presumption was proved incorrect when in 1997 the H5N1 avian influenza virus infected and killed several humans. Since then a number of studies have been performed aiming to explain the ability of avian influenza viruses to infect humans. Human influenza virus receptors are mainly expressed in the upper respiratory tract, whereas avian influenza virus receptors are primarily expressed in the lower respiratory tract (type II alveolar cells) (Figure 1I).[26,27,96] However, avian influenza virus receptors have also been detected on epithelial cells in human tracheobronchial cell cultures and in human tissue sections of trachea and bronchi, albeit to a lesser extent than human influenza virus receptors.[92,93,97–100] This may explain the capability of the virus to infect humans. In addition, H5N1 viruses are capable of infecting *ex vivo* nasopharyngeal tissues, despite a limited number of avian influenza virus receptors detected.[26] Therefore, it has been suggested that the H5N1 virus may also use alternative binding sites on the epithelium to enter target cells.[27]

Conflicting results have been reported as to the cell type expressing avian influenza virus receptors in the trachea and bronchi. Some studies have found such receptors to be located mainly on basal cells[101] or only on goblet cells,[91,92] whereas others have detected their presence primarily on ciliated cells[97–100] and only on a small proportion of nonciliated cells,[98–100] including basal cells.[99] In tracheobronchial cell cultures avian influenza viruses have been found to infect mainly ciliated cells.[97,98,102] Alveolar macrophages appear to have none or very few avian influenza virus receptors.[27,101]

The receptor distribution in extra-pulmonary tissues has been less extensively studied. Thus far neurons and the epithelial cells of the pancreatic and bile ducts have been found to express avian influenza receptors.[103,104] In addition, avian influenza virus receptors have been detected on endothelial cells in many organs throughout the body.[101] Some studies have reported the presence of avian influenza virus receptors on the epithelial cells of the intestinal mucosa,[105,106] whereas others did not find their presence on such cells.[101] With respect to immune cells, avian influenza virus receptors have been detected on T cells[87] and Kupffer cells of the liver.[101] The receptor distribution pattern as detected by lectin histochemistry broadly resembles that of infected organs and cells as demonstrated by *in situ* hybridization. However, the abundant expression of avian influenza virus receptors found on the endothelial cells of various organs contrasts with the absence of virus in these cells. In addition, the widespread and abundant expression of avian influenza virus receptors in the lungs is not in line with the limited number of infected pneumocytes, as detected by *in situ* hybridization/IHC.[7] At the same time the absence of avian influenza virus receptors on placental macrophages, alveolar macrophages, cytotrophoblasts, and intestinal

epithelial cells is inconsistent with the detected infection of such cells. These discrepancies further support the assumption that other receptors, co-receptors, or mechanisms may play a role in the interaction between the virus and its target cells, thus warranting further investigation.

Receptor Switch and Human-to-Human Transmission

Most avian and human H5N1 isolates only bind to avian influenza virus receptors.[67,70,71] Only a limited number of H5N1 human isolates have been identified that are capable of binding to human influenza virus receptors *in vitro*.[35,71,96,97] It is thought that for efficient human-to-human transmission the HA protein of influenza viruses should preferentially recognize human influenza virus receptors.[96,107,108] Previous studies with H1, H2, and H3 serotypes have demonstrated that minor mutations in the HA gene may cause receptor specificity to switch from recognizing avian influenza virus receptors to recognizing human influenza virus receptors.[94,95,109,110] Similar mutations have been introduced on the framework of an A/Vietnam/2004 H5N1 virus.[108] Mutations enabling H1 serotypes to recognize human receptors applied to H5N1 virus affect its affinity for avian receptors but do not result in human receptor specificity. In contrast, mutations enabling H2 and H3 receptors to recognize human receptors applied to H5N1 virus resulted in significant binding of the mutant virus to a natural, branched α-2,6-linked biantennary N-linked glycan and in a reduced binding to α-2,3-linked SA receptors. Viruses with these properties would be able to evade the virus-neutralizing effects of mucins containing α-2,3-linked SAs and bind more avidly to lung epithelial cells expressing α-2,6-linked biantennary N-linked glycans.[108] Yamada and colleagues[107] have recently provided further insight in possible mutations affecting the ability of the H5N1 virus to bind to human receptors. By performing reverse genetics studies and crystal structure determination, they have identified two mutations at position 182 and 192 of HA that enhance binding of H5N1 viruses to human influenza virus receptors. Despite the capability of a number of human H5N1 isolates to bind to human influenza virus receptors *in vitro*,[35,71,96,107] these isolates do not spread efficiently from human-to-human *in vivo*. Animal experiments with reassortant viruses have also shown that the mere acquisition of human influenza surface proteins does not necessarily confer transmissibility of H5N1 virus.[111] Inoculation with a reassortant virus containing genes for internal proteins of H5N1 A/Hk/486 and for surface proteins of human H3N2 A/vic/75 did not result in efficient transmission among ferrets in a respiratory droplet experimental design, even though viral replication was not compromised.[101] In addition, four H5N1 human isolates (A/Vn/1203/04, A/Vn/JP36-2/05, A/Hk/213/03, and A/Turkey/65-596/06), including isolates capable of binding to human influenza virus receptors (A/Hk/213/03 and A/Turkey/65-596/06), have been studied in a direct contact model of ferrets.[35] No transmission of either H5N1 A/Vn/1203/04 or A/Turkey/65-596/06 virus was detected. Although transmission of both H5N1 A/Hk/213/03 and A/Vn/JP36-2/05 viruses occurred, it appeared to be far from efficient. No secondary transmission from an infected contact ferret to a naïve contact ferret was demonstrated. On the basis hereof it appears that increased binding affinity for human influenza receptors alone is not sufficient for efficient transmission. Additional molecular determinants seem to be required. It has been suggested that certain biological properties such as the capacity to induce virus excretion from the upper respiratory tract (coughing and sneezing) may enhance efficient transmission.[111] As mentioned above, the presence of lysine at position 627 is a molecular determinant associated with such a capacity and may, therefore, contribute to efficient spread among humans. However, it seems likely that various additional amino acid changes are required to give avian influenza viruses the capacity to spread among humans.[83] In fact for the 1918 H1N1 influenza virus, a human influenza virus that is supposed to be derived solely from an avian source, various amino acid changes differentiate the human isolate from its avian consensus sequences.[84,112]

Implications on Receptors

The widespread distribution of avian influenza virus receptors in various organs may explain the multiple organ involvement seen in H5N1-infected humans. However, there are a number of discrepancies between the cell types expressing avian influenza receptors and the cell types found to be infected by the H5N1 virus. Despite the relative lack of avian influenza virus receptors, viral replication has been demonstrated in the upper respiratory tract. Therefore, further research is needed to investigate the possible role of other receptors or mechanisms involved in the interaction between the virus and its target cells. The identification of other receptors could help the design of effective drugs treating H5N1 infection or preventing transmission. Recent studies have shown that not only the acquisition of the capacity to bind to human influenza virus receptors but also other genetic changes may be necessary for efficient transmission among humans. Continuous surveillance of the circulating H5N1 strains is of key importance as the emergence of amino acid substitutions similar to those demonstrated for the 1918 H1N1 virus might indicate that the virus could acquire pandemic potential in the near future.

Final Remarks

Since 1997 several studies have contributed to fundamental insights into the pathology and pathogenesis of human H5N1 influenza. Aside from the respiratory tract, other organs such as the intestines, the brain, and the placenta appear to be infection targets of the virus. The H5N1 virus is also capable of transplacental transmission to the fetus. Dysfunction of the immune system may be a key pathogenetic mechanism. At the molecular level, several viral genes and mutations in gene products have

been suggested to be involved in increased virulence of H5N1 viruses. At the same time, however, it becomes increasingly apparent that what is known today about the virus and its pathogenicity is only the tip of the iceberg and that there are likely several additional pathogenetic mechanisms and molecular determinants of pathogenicity in H5N1 influenza yet to be identified. In light of the subsisting threat of a potentially devastating influenza pandemic, further investigations in these respects are urgently required.

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