Med Microbiol Immunol (2007) 196:191–201
DOI 10.1007/s00430-007-0045-2

REVIEW

The threat of avian influenza a (H5N1): part II: Clues to pathogenicity and pathology

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Received: 9 March 2007 / Published online: 4 April 2007

Abstract Among emerging and re-emerging infectious diseases, influenza constitutes one of the major threats to mankind. In this review series epidemiologic, virologic and pathologic concerns raised by infections of humans with avian influenza virus A/H5N1 are discussed. The second part focuses on experimental and clinical results, which give insights in the pathogenic mechanisms of H5N1 infection in humans. H5N1 is poorly transmitted to humans. However, H5N1-induced disease is very severe. More information on the role entry barriers, H5N1 target cells and on H5N1-induced modulation of the host immune response is needed to learn more about the determinants of H5N1 pathogenicity.

Introduction

The big outbreak of H5N1 infection in a chicken mass holding farm of Hong Kong in 1997 resulted in virus transmission to 18 humans causing six deaths [1, 2]. Although within 10 years after the outbreak the avian H5N1 virus has not yet mutated to cause a human pandemic, we believe that the risk remains constant as long as virus continues to circulate and H5N1 viruses with increasing pathogenic potential evolve [3]. Highly pathogenic H5N1 viruses have already infected an increasing number of humans, resulting in high mortality rates and the emergence of multiple distinguishable clades [4]. Currently, only limited information on pathogenicity and pathology of H5N1 infection in humans is available. H5N1 infection of humans is mediated by many factors including virus ability to penetrate entry barriers of respiratory tract and to adapt on specific virus receptors on target cells. Notably, H5N1 still did not adapt to the first steps of infection in humans. However, clinical manifestation is much more severe after virus infection of target cells in the respiratory tract when compared with human adapted influenza A viruses causing seasonal flu. High viral load is central to pathogenesis suggesting that tissue damage in respiratory tract may be largely due to direct lysis of infected cell [5]. A limitation of infection to respiratory and intestinal tract [6, 7] suggests that additional mechanisms are involved in a severity of the disease. Similar to H1N1 influenza strain causing the 1918 pandemic, H5N1 viruses are able to dysregulate inflammatory and immune responses, which may contribute significantly to severity and high mortality associated with the H5N1 infection in humans [8–10]. In this review, we discuss results of cell culture and animal experiments as well as clinical investigations, which give some new insights in the pathogenic mechanisms of H5N1 infection in humans. Major determinants of different responses during infection with human adapted and avian influenza A virus strains are given in Table 1.

Interactions with anatomical entry barriers

To infect a sensitive host, influenza viruses must break mucous membranes of the respiratory tract tissues where a virus must attach to host cell receptor and enter epithelial cells before it can replicate. Mucus represents one of the important barriers in the respiratory system and is produced by submucosal cells as well as goblet cells. Foreign particles

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Table 1 Major determinants of different responses during infection of humans with human adapted and avian influenza A H5N1 strains

Human-adapted influenza A (H3N2, H1N1)	Avian influenza A (H5N1)
Effective breaching of entry barriers in the respiratory tract	Limited breaching of entry barriers
Infection throughout the respiratory tract	Preferential infection in the lower respiratory tract
Limited virus replication in the lungs and dissemination	High and rapid replication in the lungs; dissemination in the intestinal tract and in the central nervous system (?)
Activation of normal innate and adaptive immune responses	Dysregulated innate and adaptive immune responses
Transient hypercytokinemia and hyperchemokinemia	Persistent (very) high cytokine and chemokine levels
Transient lymphopenia without alteration in CD4+ to CD8+ ratio	Prolonged lymphopenia with inverted CD4+ to CD8+ ratios

are removed from the nasal cavity or upper respiratory tract by trapping in mucus, carrying them back to the throat and by swallowing. From the lower respiratory tract foreign particles are cleared by the ciliary action of epithelial cells. In the alveoli that lack cilia or mucus, foreign particles are phagocytosed by macrophages. Mucus consists of mucins, highly glycosilated peptides (70–80% per weight), which form low molecular mass monomeric and high molecular mass dimeric or oligomeric mucins [11]. A major function of the secretory mucins is to act as a microbe/debris removing multimeric network that harbours defence molecules and holds fluid in space. Chimpanzees are relatively resistant to experimental respiratory exposure to human influenza A viruses, possible because mucins in their respiratory tract secretions can specifically bind viruses [12–14]. This selective binding may be explained by the presence of α2,6-linked sialic acid (α2,6 SiA) in secreted mucins which are preferentially used by human influenza A viruses as cellular receptors (see below). Consequently mucins in respiratory tract of chimpanzees could inactivate human influenza A viruses (through α2,6 SiA binding) before they reach the layer of epithelial cells. During the evolution, humans have undergone a bi-directional switch in SiA expression between airway epithelial cell surfaces from α2,3-linked sialic acid (α2,3 SiA) to α2,6 SiA and secreted mucins from α2,6 SiA to α2,3 SiA [14]. Since avian influenza A viruses use preferentially α2,3 SiA as a cellular receptor (see below), mucins secreted in human respiratory tract could specifically bind and inactivate avian influenza A viruses, which may explain why humans are relatively resistant to infection with H5N1 strains [15]. Human corneal and epithelial cells express predominantly α2,3 SiA whereas ocular secretory mucins are rich in α2,6 SiA [15]. The selectivity of SiA linkage on cells and in secretions of the eye may explain the ocular tropism exhibited by avian influenza A viruses. Conjunctivitis was observed in the outbreak of avian H5N1 in Hong Kong in 1997, H7N7 in The Netherland in 2003, H7N2 in USA in 2003 and H7N3 in Canada in 2004 [15].

Influenza viruses developed mechanisms to overcome respiratory tract protection of mucins. Enzymatic function of neuraminidase (NA), a surface glycoprotein of influenza A, was shown to be involved in penetration of the virus through mucosa by cleaving sialic acid associated with mucins, which would impede virus access to functional receptors on target cells [16–18]. In addition, it has been hypothesized that NA could cause disruption of the mucosa–IgA axis, creating localized partial immunosuppressed state, enhancing both influenza infection itself and secondary bacterial pneumonia [19]. This condition may be achieved by enzymatic removing of sialic acid from IgA, consequently disrupting the normally existing homeostasis of IgA in the host [19]. It is well established that mutations in NA gene may be associated with changes in its activity [20]. In H1N1 virus, which caused Spanish influenza pandemic of 1918–1919 mutations in NA gene contributed to high pathogenic potential of the virus [21–23]. Large-scale sequence analysis of avian influenza H5N1 isolates showed that NA together with HA and NS1 contribute the most to the variability of avian virus genome [24]. H5N1 viruses with amino acid deletion in NA stalk may exert changes in biological properties with expanded host range [25, 26]. It remains to be elucidated whether effects of H5N1 NA on respiratory tract mucins may play a role in pathogenesis of avian influenza in humans.

Other molecules such as innate defence proteins produced in lung may hinder influenza viruses to reach epithelial cells and therefore also provide protection. For example, surfactant protein-D (SP-D) secreted by alveolar type II cells and by non-ciliated Clara cells was shown to inactivate human influenza A strains [27]. It has been demonstrated that a common human polymorphic form of SP-D may modulate defence against influenza A virus [28]. The common Thr/Thr11 polymorphism is associated with assembly predominantly as trimers, whereas the more common forms of SP-D are assembled into dodecamers or higher molecular weight multimers. Natural trimeric SP-D has reduced ability to bind and to neutralize influenza A viruses [28]. On the other hand, human influenza A viruses which are resistant to inactivation by SP-D were reported [27]. These findings suggest that the degree of protection of the entry barrier of the respiratory tract against influenza

infection depends both on host and virus genotype. Antiviral protective activity of the single constituents of the respiratory tract entry barrier (e.g. respiratory tract mucins, and surfactant proteins) should be studied in the view of H5N1 infection.

Target cells and tissue tropism

Human influenza viruses replicate through entire the respiratory tract, with virus being recoverable from the upper respiratory tract and lower respiratory tract. Human influenza virus specific antigen is present in types I and II alveolar epithelial cells, as well as in intraalveolar macrophages [29]. Human adapted viruses bind extensively to epithelial cells in the bronchi and, to a lesser degree, to alveolar cells [30]. In contrast, H5N1 virus preferentially infects cells in the lower respiratory tract where it attaches predominantly to type II pneumocytes, alveolar macrophages and non-ciliated cuboidal epithelial cells in terminal bronchioles [30, 31]. The cells of the whole respiratory tract harbour the α2,6 SiA receptor for human adapted influenza viruses while the cells of the lung alveoli express also the α2,3 SiA receptor for avian influenza viruses as was demonstrated by experimental adsorption of laboratory strains of H5N1 virus to alveolar epitheliocytes in histological sections [30, 31]. However, one human H5N1 virus isolate (A/Hong Kong/213/03), which recognizes both α2,3 SiA and α2,6 SiA was shown to bind extensively to both bronchial and alveolar cells [30]. The infection of type-II pneumocytes and alveolar macrophages with H5N1 viruses may contribute to the severity of pulmonary lesions [31]. Damage to metabolic active type-II pneumocytes may impair their function, including re-epithelization after alveolar damage, ion transport and surfactant production. This may result in inhibition of tissue repair. Infection of macrophages may compromise their function in innate immune reactions and aggravate inflammatory responses to H5N1 infection.

The attachment of H5N1 was also studied in respiratory tract tissues from several animal species. The pattern of H5N1 virus attachment to cat lower respiratory tract and, to a lesser extent, ferret lower respiratory tract most closely resembles that in human tissues [31]. Susceptibility of cats to avian influenza was demonstrated by observations of lethal infection in wild and domestic cats, who were fed with H5N1 contaminated chicken meat [32] or in a domestic cat by eating a pigeon carcass [33]. A recent study demonstrated that α2,3 SiA but not α2,6 SiA is preferentially expressed in the mouse lung [34]. However, in mice the H5N1 virus attachment to cells was most abundant in the trachea and became progressively rarer towards the alveoli, whereas opposite trend was observed in human tissues [31]. In macaque's alveoli, H5N1 virus attached predominantly to type-I pneumocytes in contrast to humans in which H5N1 virus attached predominantly to type-II pneumocytes. The observed pattern of H5N1 virus attachment to the lower respiratory tract is consistent with the respective pathology of experimental H5N1 virus infection in mice, ferrets, macaque and cats. Taken together, cat and ferret represent the most suitable animal models for human H5N1 viral pneumonia [31, 32, 35].

The restriction of H5N1 infection to human lower respiratory tract may contribute to inefficient human-to-human transmission of H5N1 viruses seen up to date. One could speculate, that if H5N1 mutates to a human-adapted virus easily adsorbing to mammalian α2,6 SiA receptor, infectivity of this influenza virus will dramatically increase, due to ability of the virus to replicate in upper respiratory tract and easy spread to another host by sneezing and coughing. This notion is supported by the finding that earliest isolates in 1918 [36, 37], 1957 and 1968 pandemic preferentially recognized α2,6 SiA [38], even though their HAs were derived from avian viruses. Point mutations ("antigenic drift") may be responsible for the adaptation of H5N1 virus to humans. The change of one amino acid of the H5 protein could be potentially sufficient to change the receptor specificity of H5N1 viruses [39]. In addition, H5N1 viruses might overcome their inefficient transmission to humans by reassortment with human viruses ("antigenic shift") as was the case for the pandemics of 1957 and 1968 caused by avian-human reassortant influenza viruses [40]. In a comparative ferret model that parallels the efficient transmission of H3N2 human viruses and the poor transmission of H5N1 avian viruses in humans, reassortants of human and avian influenza viruses were studied [41]. An H3N2 reassortant virus with avian virus internal protein genes exhibited efficient replication but inefficient transmission, whereas H5N1 reassortant virus with four or six human virus internal protein genes exhibited reduced replication and no transmission. These findings indicate that the human virus H3N2 surface protein genes alone did not confer efficient transmissibility and that acquisition of human virus internal protein genes alone was insufficient for the H5N1 virus (A/Hong Kong/486/97 strain) to develop pandemic capabilities, even after serial passages in a mammalian host. These finding suggest that reassortant viruses would likely need more genetic changes, such as ones that make the 1957 and 1968 strains better able to bind to human respiratory tract epithelial cells.

Moreover, recent investigations demonstrate that ex vivo cultures of human nasopharyngeal, adenoid and tonsillar tissues can be infected with H5N1 viruses in spite of an apparent lack of α2,3 SiA [42]. These findings imply that the inefficiency of the avian-to-human or human-to-human transmission of the H5N1 virus may not be explained by the inability of the virus to replicate in these sites. Virus infection of cells that apparently do not express α2,3 SiA

implies that there may be other binding sites on the epithelium that mediate virus entry. Furthermore, because human H1N1 and avian H5N1 viruses do not differ in their ability to replicate in the alveolar epithelium, it may be also concluded that the increased severity of human H5N1 influenza cannot be explained purely on the basis of a differential tropism of H5N1 to the lower respiratory tract epithelium [42]. These findings are also of interest in the view of tissue tropism of H5N1 viruses in humans. In one case, viral RNA was detected in lung, intestine and spleen by a reverse transcription polymerase chain reaction, but positive-stranded viral RNA, indicating virus replication, was confined exclusively to the lung and intestine [6]. Thus, H5N1 viral replication in humans may be restricted to the respiratory and intestinal tract in contrast to disseminated infections documented in other mammals and birds. However, replication in other sites such as CNS cannot be excluded since the infectious virus and RNA was detected in cerebrospinal fluid of a child with H5N1 infection [5]. It should be also noted that the site of inoculation can determine the pathway of spread of the influenza virus in the host. Animal studies have revealed that the neurotropic influenza A NWS strain disseminates to the brain by haematogenous spread when given intraperitoneally but reaches the CNS via the sensory neurons when the virus inoculum is placed into the nose [43]. The virus invasion in the CNS was also observed for H5N1 strain (A/Hong Kong/483/97) after intranasal virus inoculation in mice [44].

Immune dysregulation and inflammation

Once influenza virus has efficiently infected respiratory epithelial cells, replication occurs within hours and numerous virions are produced. Infectious particles are preferentially released from the apical plasma membrane of epithelial cells into the airways by a process called budding. This favours the swift spread of the virus within the lungs due to the rapid infection of neighbouring cells. In an uncomplicated influenza virus infection, the cytopathic effect of viral infection is most often observed as degenerative and necrotic changes in epithelial cells of the bronchial and bronchiolar mucosa [29]. Severe influenza infections are associated with significant changes in alveolar cells, typified by acute and focal alveolitis [29]. Inflammation is often characterized by a rapid course of infection, with death resulting within days of the onset of clinical symptoms [29]. In humans with fatal H5N1 infection post-mortem analyses have documented extensive pneumocyte type-II destruction with severe pulmonary injury and histopathological changes of diffuse alveolar damage [7, 45]. The development of acute respiratory distress syndrome occurred at the end of the first week (median time, day 6).

The pathogenicity of H5N1 viruses in animals seems to be determined by high replication efficiency, broad tissue tropism and systemic replication [35, 46, 47]. These features of avian influenza infection may be true at least in part for humans. H5N1 infection in patients in Vietnam during the years 2004 and 2005 and Indonesia during the year 2006 was characterized by high viral loads in the throat and nose [48, 49] and frequent detection of viral RNA in rectum and blood [48]. Viral RNA in blood was present only in fatal H5N1 cases and was associated with higher pharyngeal viral loads [48]. Moreover, low peripheral blood T-lymphocyte counts and high chemokine and cytokine levels correlated with pharyngeal viral loads, particularly in H5N1-infected individuals who died [48]. These observations indicate that high viral load, and the resulting immune dysregulation and inflammatory responses, are central to H5N1 pathogenesis.

Influenza A viruses may influence antiviral immune responses by interaction of HA protein with innate and adaptive immune mechanisms. A recombinant HA protein from H5N1 was shown to suppress perforin expression and reduce cytotoxicity of human CD8+ T cells to kill H5N1-virus- or H5-bearing cells [50]. These effects of HA may reduce ability of CD8+ T cells to limit virus replication in epithelial cells of respiratory tract. In addition, decreased specific cytolytic activity of CD8+ T cells may enable persistence of H5-presenting dendritic cells providing sustained antigenic stimulation to CD8+ T cells [50]. The persistent activation of CD8+ T cells leads to hyperproduction of IFN-γ, which may result in overactivation of macrophages and subsequent hyperproduction of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), a feature similar to that found in H5N1 infected humans. Since HA protein from H1N1 or H3N2 viruses did not show such effects, it may be hypothesized that the manifestation of severe infection by H5N1 viruses may be associated with the insufficient perforin expression [50]. HA of influenza A viruses was also shown to bind to NK cell-specific activating receptors NKp46 (also called NCR1) and NKp44 belonging to the group of natural cytotoxic receptors (NCRs) [51, 52]. Interactions of NCRs with HA are mediated largely via sialylated residues of NKp44 and NKp46 and result in NK cell activation and enhanced killing of influenza infected cells. NKp46 was demonstrated in animal experiments to play a critical role in innate immune response to influenza A virus infection [53]. To explore NKp46 functions, a homozygous mutant of a mouse was generated in which NKp46 was replaced with a green fluorescent protein reporter cassette. More than half of the control mice survived influenza virus infection after intranasal inoculation, whereas all of the homozygous mutants succumbed, even though NK cells accumulate in the lungs. In influenza virus infected respiratory mucosa NK cells may

become activated not only after NKp46 binding of HA on epithelial cells, macrophages and dendritic cells but might involve cooperative signalling by other activating NK cell receptors such as NKG2D triggered by induced ligand expression in infected cells [54, 55]. Activated NK cells may in turn activate dentritic cells by receptor ligand interactions, thus promoting maturation of dendritic cells while influenza-infected dendritic cells attract NK cells through a coordinated programme of chemokine secretion [56]. The cross-talk between NK cells and dentritic cells promotes dentritic cell maturation and subsequent T cell priming. However, overactivation of NK cells may lead to excessive production of pro-inflammatory cytokines and chemokines, which could contribute to the severe pathogenesis of H5N1 infection. A transient increase in NK cell activity (but not NK cell numbers) was measured in some individuals after human influenza virus infection [57, 58]. A comparison of influenza A (H3N2) infected patients with normal NK cell activity and those with increased NK cell activity at admission showed longer recovery time in patients with higher NK cell activity [58].

One of the major mechanisms of immune dysregulation and inflammatory responses during influenza infection may result from activity of the NS1 protein. The NS1 protein is a bifunctional viral immunosuppressor, which inhibits both innate and adaptive immunity. The suppression of adaptive immunity by influenza A virus was demonstrated by findings that infected human dendritic cells have lower allospecific Th-1 stimulatory abilities than dentritic cells activated by other stimuli, such as lipopolysaccharide and Newcastle disease virus infection [59]. This weak stimulatory activity correlates with the suppression of the genetic programme underlying dendritic cell maturation, migration and T-cell stimulatory activity through specific activity of NS1 protein. The suppressive activities of NS1 on T cell immune responses were demonstrated for several H1N1 and H3N2 influenza A strains. However, it remains to be elucidated whether the NS1 protein of H5N1 virus exerts similar effects on adaptive immune responses.

The NS1 protein of highly pathogenic H5N1 viruses subverts innate immune responses due to its ability to confer resistance to the antiviral effects of interferons (IFNs) and TNF-α. Pretreatment of porcine lung epithelial cells with IFN-α, IFN-γ, or TNF-α had no effect on the replication of a recombinant human H1N1 virus possessing NS1 gene of the 1997 H5N1 but abolished replication of the parental human H1N1 virus [60, 61]. Resistance to the antiviral effects of IFN and TNF-α is associated with the presence of glutamic acid at position 92 (Glu92) of the NS1 protein, as demonstrated by reverse genetic studies. These in vitro data extend to in vivo findings, since pigs infected with a virus containing Glu92 in NS1 experienced higher virus titers and body temperatures than those infected with a control virus [60, 61]. Viral strains from different animal hosts likely have NS1 genes adapted to antagonize the IFN-α/β system of their specific host species [62]. If this is a general property of NS1 from different influenza virus strains, an avian strain would require adaptation of its NS1 gene to efficiently antagonize the human IFN-α/β system. Interestingly, molecular analysis of human H5N1 isolates from Vietnam did not reveal Glu92 in the NS1 protein, but all isolates contained a sequence motif, Glu-Ser-Glu-Val (ESEV) in the carboxyl terminus of the NS1 protein [24, 48]. These residues are predicted to mediate binding to proteins bearing a region called a PDZ domain. The multitudes of human proteins that contain a PDZ domain function in diverse cellular signalling pathways including those that regulate cytokine expression. Functional studies demonstrated that the C terminus of NS1 in low pathogenic human influenza viruses does not interact with PDZ domains [24]. It is therefore important to investigate a functional role of ESEV motif in NS1 protein of H5N1 viruses in terms of its effects on IFN expression and activity.

Highly pathogenic H5N1 viruses are not only resistant to antiviral effects of IFN and TNF-α but also trigger the overproduction of pro-inflammatory cytokines. The H5N1/97 virus was a potent inducer of pro-inflammatory cytokine genes, particularly TNF-α and IFN-β in human primary monocyte-derived macrophages [63, 64]. The precursor of H5N1/97 virus H9N2/G1, which shares six internal segments with H5N1/97, also shared high cytokine induction phenotype [63]. Although recombinant viruses containing NS gene (coding for NS1 and viral nuclear export protein) of the H5N1/97 virus induced expression of TNF-α in human macrophages, the cytokine production was lower than that induced by wild-type H5N1 virus. These results suggest that internal genes or gene products other than NS gene contribute to the manifestation of the high-TNF-α phenotype [63]. Interestingly, induction of TNF-α contributed to morbidity during H5N1/97 infection in a mouse model, while interleukin 1 (IL-1) was important for effective virus clearance in non-lethal H5N1 disease [65]. Hyperinduction of TNF-α in primary human macrophages in response to H5N1/97 virus involves the p38 mitogen-activated protein kinase signalling pathway [66]. Similarly, 2003 and 2004 human H5N1 isolates induce high levels of pro-inflammatory cytokines in primary human macrophages [67] as well as in primary human alveolar and bronchial cells [68] relative to infection with H1N1 viruses. Some of the cytokines such as IP-10 (IFN-γ-inducible protein-10; CXCL10) appeared to be induced more potently by recent H5N1 viruses from Vietnam (H5N1/04) than by H5N1/97 virus [68]. Moreover, significantly higher expression of chemokines including MCP-1 (monocyte chemoattractant protein 1; CCL2), MIP-1α (macrophage inflammatory protein 1; CCL3) and RANTES (regulated on

activation, normal T cell expressed and secreted; CCL5) and chemokine receptors CCR1 and CCR5 was induced in monocyte-derived macrophages infected with H5N1/97 virus than with human H1N1 virus [69]. The induction of pro-inflammatory cytokines and chemokines may depend on the cell type infected. In human type II pneumocytes different H5N1 virus strains failed to induce IL-8, MCP-1 and TNF-α [68], while these pro-inflammatory mediators were strongly induced by H5N1 viruses in human monocyte-derived macrophages [64, 69].

There is only limited information on production of pro-inflammatory cytokines and chemokines in H5N1-infected humans (Table 2). In 1997 outbreaks, elevated blood levels of interleukin-6, TNF-α, IFN-γ and soluble interleukin-2 receptor were observed in individual patients [8]. In patients from a 2003 outbreak, elevated levels of the chemokines IP-10 and MIG (monokine induced by IFN-γ; CXCL9) were found 3–8 days after onset of illness [9]. The relevance of cytokine and chemokine dysregulation for severity of H5N1 infection was also assessed in 18 individuals infected in Vietnam during the years 2004 and 2005 and was correlated with pharyngeal virus loads [10]. The levels of proinflammatory proteins measured in peripheral blood including IP-10, MIG, MCP-1 and IL-10 were elevated in patients with avian and human subtypes of influenza, but were higher in H5N1-infected individuals and particularly high in those who died. Levels of IL-8 were also elevated in H5N1-infected individuals (particularly those who died) but not in humans infected with H3N2 virus. The plasma levels of IP-10, MCP-1, IL-8, IL-6 and IL-10 correlated with pharyngeal H5N1 load. This indicates that the observed hypercytokinemia and hyperchemokinemia reflect, at least partly, increased viral replication [10]. It should be noted that a measurement of cytokines and chemokines in blood of H5N1 infected humans may reflect the events in the highly vascularized lung. However, levels of cytokines in lung tissue may be more relevant to pathogenesis of respiratory infections than levels in blood [70].

A dysregulation of pro-inflammatory responses in patients with H5N1 infection may promote a development of secondary haemophagocytic lymphohistiocytosis (HLH) [71]. Investigators from Hong Kong have reported that two patients with fatal H5N1 disease in 1997 had a reactive haemophagocytic syndrome as the most prominent feature [8]. Reactive haemophagocytosis has also subsequently been reported in H5N1-infected patients from Hong Kong in 2003 and from Thailand in 2004 [9, 72]. There are clinical similarities between H5N1 infection and HLH (familial or Epstein-Barr-virus-associated HLH), such as massive hypercytokinaemia, cytopenia and acute encephalitis. The cause of death in patients with HLH is often a sepsis-related condition with multiorgan failure, which is also seen in patients with fatal H5N1 infection. HLH triggered by other severe viral infections such as Epstein-Barr-virus is associated with high mortality, which may be decreased (from 50 to 10%) if specific HLH therapy is involved [71]. A mechanism for HLH therapy is probably a down-regulation the inflammatory response by apoptosis-triggering of overactivated cells within the immune system [73]. It would be therefore of interest to show whether such a strategy would help to manage dysregulated immune responses in humans infected with H5N1 viruses [71].

Table 2 Chemokine and cytokine levels in the peripheral blood of H5N1 infected humans

Chemokine/ cytokine	Hong Kong		Ho Chi Minh City 2004/2005 (n = 18)
	1997 (n = 2)	2003 (n = 2)	
IFNγ	Transient increase[a]	–	H5N1 > H3/H1[b]
TNFα	Increase in one patient	Undetectable	–
IL-β	–	Undetectable	–
IL-6	Sustained increase	Low increase in one patient[d]	H5N1 > H3/H1 Correlation with pharyngeal virus load
IL-8	–	Similar levels	H5N1 > H3/H1 Fatal > survivors[c] Correlation with pharyngeal virus load
IL-10	–	Undetectable	H5N1 > H3/H1 Correlation with pharyngeal virus load
IP-10	–	Increased	H5N1 > H3/H1 Fatal > survivors Correlation with pharyngeal virus load
MCP-1	–	Similar levels	H5N1 > H3/H1 Fatal > survivors Correlation with pharyngeal virus load
MIG	–	Increased	H5N1 > H3/H1 Fatal > survivors
RANTES	–	Similar levels	–

[a] Compared with serum levels of healthy controls

[b] Compared with serum from patients with uncomplicated influenza A H3N2 or H1N1 strains

[c] Serum levels were significantly higher in fatal cases than in survivors of H5N1 infection

[d] Compared with serum from patients with uncomplicated influenza A or B

Notably, lymphopenia was observed in H5N1-infected patients and the clinical severity of human H5N1 disease correlated with low peripheral white blood cell and lymphocyte counts at admission [1, 7, 74–77]. Measurement of peripheral blood lymphocyte counts in patients in Vietnam during the year 2004 and 2005 showed low total and CD3+ lymphocyte counts and inverted ratios of CD4+ to CD8+ cells particularly in H5N1-infected individuals who died [10]. This feature of H5N1 disease is in contrary to infection with human adapted influenza A viruses when lymphopenia is found without alteration in CD4+ to CD8+ ratio [58, 78]. Moreover, total and CD3+ lymphocyte numbers in H5N1-infected individuals correlated inversely with pharyngeal viral RNA load, suggesting an association between lymphopenia and the level of viral replication. Different mechanisms may play a role in lymphopenia observed in H5N1 infected humans. In one H5N1-infected individual bone marrow hypoplasia was reported suggesting decreased production of lymphocytes [8]. A peripheral blood lymphopenia may result from trafficking of lymphocytes to infected tissue. However, post-mortem studies in H5N1-infected individuals have not shown predominance of lymphocytes, but instead of macrophages, in pulmonary infiltrates [9]. Induction of lymphocyte apoptosis by death receptor ligands including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) may play an important role in lymphopenia associated with H5N1 infection. In cell culture experiments supernatants from human monocyte-derived macrophages infected with H5N1 virus exerted a cytotoxic effect on T lymphocytes by triggering apoptosis [69]. T cell apoptosis was induced by functional TRAIL released in culture supernatants of macrophages infected with H5N1 but not by H1N1 viruses. Furthermore, H5N1/97 infection of T lymphocytes enhanced their sensitivity to apoptosis induced by TRAIL and other death receptor ligands including TNF-α and FasL [69]. Interestingly, avian influenza viruses including H5N1/97 and H9N2/G1 induced apoptosis in T cells (Jurkat T cell line) and dendritic cells to a lesser extent when compared with human virus H1N1/98 [69]. The decreased apoptosis in avian influenza virus-infected cells may contribute to the prolonged viral replication [79]. On the other hand, TRAIL and FasL have also been found to enhance the propagation of influenza A virus in epithelial cells [80]. The inhibition of apoptosis in lymphocytes and antigen presenting cells infected with H5N1 virus may be a result of antiapoptotic affects of CCL5 and CCR5 which are strongly up-regulated by H5N1 infection in human macrophages [64]. CCL5–CCR5 interaction was already shown to provide antiapoptotic signals for macrophage survival during viral infection with human influenza virus [81]. It was reported that viral induction of the apoptotic process limits the release of proinflammatory cytokines [82]. Therefore, it is possible that the limitation of apoptosis in H5N1 infected cells such as macrophages (e.g. through CCL5–CCR5 pathway) promotes the release of proinflammatory cytokines. This may enhance the severity of the inflammatory response to infection as observed in patients with avian virus infection. Numerous proteins of influenza A virus may be involved in regulation of apoptosis in infected cells [79]. NS1 protein exerts both antiapoptotic and proapoptotic activities in cultured cells depending on cell type and experimental conditions [83, 84]. M1 protein was shown to bind specifically caspase 8 suggesting interference of M1 with the caspase 8 mediated apoptosis pathway [85]. The PB1-F2 protein has been characterized as proapoptotic protein that is expressed from alternative +1 reading frame of the PB1 polymerase gene segment [86]. This alternate reading frame is found in the pandemic strains of 1968, 1957 and the infamous 1918 strain [87]. Molecular analysis of H5N1 strains showed that PB1-F2 were under positive selection pressure suggesting that this gene may play a role in H5N1 pathogenicity [88]. The PB1-F2 protein contains a mitochondrial target sequence and was shown to induce apoptosis in macrophages and lymphocytes (not in epithelial cells) by mitochondrial membrane permeabilization [86, 89, 90]. Another mode of apoptosis induction by influenza virus might occur via the activation of TGF-β. Influenza A viruses activate latent TGF-β on the cells surfaces through NA-mediated cleavage of the cytokine into its active form [91]. This mechanism may also contribute to lymphopenia since TGF-β is a potent inducer of apoptosis in lymphocytes. Taken together, the decrease in lymphocyte counts caused by different mechanisms in patients with avian influenza A viruses may cause an acute immunodeficiency that may aggravate the infection of the respiratory tract.

A significance of deregulated inflammatory and immune responses for severe pathogenicity caused by infection with high pathogenic influenza viruses was originally demonstrated using recombinant viruses containing HA and NA segments from the 1918 pandemic virus [21, 22]. In mouse studies these highly virulent recombinant viruses could infect the entire lung and induce high levels of macrophage-derived chemokines and cytokines, which resulted in infiltration of inflammatory cells and severe haemorrhage, hallmarks of the illness produced during the original pandemic [92]. Later studies using reconstructed 1918 virus demonstrated that a virus containing all eight genes from the pandemic virus induced greater host inflammatory and immune responses resulting into more severe disease pathology and accelerated death in mice when compared with viruses containing only subsets of 1918 genes [93]. The pathogenic potential of the 1918 virus was also studied in primates [94]. The 1918 virus caused a highly pathogenic respiratory infection in a cynomolgus macaque model that culminated in acute respiratory distress and a fatal out-

come. The infected animals mounted an immune response, characterized by dysregulation of the antiviral response that was insufficient for protection, indicating that atypical host innate immune responses may contribute to lethality. This uncontrolled immune response observed in infected monkeys resulted in the expression of abnormally high levels of cytokines and chemokines. The most striking elevation was observed in the levels of IL-6, which increased 5–25-fold by 8 days post-infection in monkeys injected with the 1918 influenza virus [94]. The plasma levels of IL-6 were also increased in humans infected with H5N1 virus (particularly those who died) and correlated with pharyngeal virus load [7, 10]. Taken together, the modulation of host immune responses seems to be critical determinant of the severity and outcome of infection by the 1918 virus and may be shared by the virulent influenza viruses such as H5N1.

Conclusion

The knowledge about H5N1 pathogenicity and pathology in humans is very limited. The antiviral protective activity of the single constituents of the respiratory tract entry barrier (e.g. respiratory tract mucins, surfactant proteins) needs to be further studied in the view of H5N1 infection. In contrast to human-adapted influenza A strains, which infect the cells of the upper and lower respiratory tract, H5N1 preferentially infects cells of the lower respiratory tract in humans, which may limit human-to-human transmission. Nevertheless, H5N1 replication results in higher viral loads and increased tissue damage when compared to human-adapted influenza A strains. Moreover, while infection with human-adapted influenza A strains is usually limited to the respiratory tract, H5N1 may spread to other tissues such as intestine or CNS once the infection has been established in humans. This virus spreading may contribute to the severity of H5N1-induced disease. More information about H5N1 target cells and tissue tropism is needed to receive a clearer picture. Parallels between H5N1 and the 1918 H1N1 virus suggest that the modulation of host immune responses may be critical for the severity and outcome of infection.

Acknowledgements The authors thank Rouslan Kotchetkov for critical reading of the manuscript. The authors have been supported by the European Commission-funded projects LSH-CT-2004-512054 (Contract number: 512054) and COOP-CT-2004 (Contract number: 512864).

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