

Pathology of Fatal Human Infection Associated With Avian Influenza A H5N1 Virus

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Eighteen cases of human influenza A H5N1 infection were identified in Hong Kong from May to December 1997. Two of the six fatal cases had undergone a full post-mortem which showed reactive hemophagocytic syndrome as the most prominent feature. Other findings included organizing diffuse alveolar damage with interstitial fibrosis, extensive hepatic central lobular necrosis, acute renal tubular necrosis and lymphoid depletion. Elevation of soluble interleukin-2 receptor, interleukin-6 and interferon- γ was demonstrated in both patients, whereas secondary bacterial pneumonia was not observed. Virus detection using isolation, reverse transcription-polymerase chain reaction and immunostaining were all negative. It is postulated that in fatal human infections with this avian subtype, initial virus replication in the respiratory tract triggers hypercytokinemia complicated by the reactive hemophagocytic syndrome. These findings suggest that the pathogenesis of influenza A H5N1 infection might be different from that of the usual human subtypes H1-H3. **J. Med. Virol. 63:242–246, 2001.** © 2001 Wiley-Liss, Inc.

KEY WORDS: emerging infection; hemophagocytic syndrome; hypercytokinemia

INTRODUCTION

Influenza A H5N1 infection was known previously to be confined to avian species [Webster et al., 1992]. During May through December 1997, 18 cases of human H5N1 infection were identified in Hong Kong.

Eight patients had mild upper respiratory illnesses, four patients had severe pneumonia requiring respiratory support but eventually recovered, and six patients died with multiple organ failure despite of intensive care. The high mortality and the potential of an emerging influenza pandemic were alarming. While underlying cardiovascular, pulmonary and renal diseases, alcoholism and pregnancy have been regarded as the major predisposing factors for severe influenza A infection [Kilbourne et al., 1995], none of these were found among the severe cases of H5N1 infection [Yuen et al., 1998]. Also, no evidence of secondary bacterial pneumonia, which has been reported as the leading cause of death in previous influenza pandemics [Kilbourne et al., 1995], was found among these severe cases. These findings suggest that the pathogenesis leading to a fatal outcome of avian influenza A H5N1 infection in humans may be different from that for the usual human subtypes H1-H3. The post-mortem findings of two fatal cases of influenza A H5N1 infection (the only cases on whom a full post-mortem was performed) are described and the possible pathogenic mechanisms leading to the poor outcome are discussed.

MATERIALS AND METHODS

Case report

In both patients, influenza A H5N1 infection was confirmed by virus isolation from respiratory speci-

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Accepted 24 July, 2000

mens. The detailed clinical findings have been described previously [Yuen et al., 1998]. Briefly, patient 1 was a previously healthy 13-year-old Chinese girl who presented with fever and flu-like symptoms for six days. Soon after admission, she developed clinical and radiological signs of right-sided pneumonia, leucopenia (total white cell count [WBC], $2.54 \times 10^9/L$; lower limit for age > 8 years, $4.5 \times 10^9/L$), lymphopenia (lymphocyte count, $0.18 \times 10^9/L$; lower limit for age > 12 years, $1.2 \times 10^9/L$), thrombocytopenia (platelet count, $31 \times 10^9/L$; lower limit, $150 \times 10^9/L$) and a slightly prolonged clotting time (activated partial thromboplastin time [APTT], 38.5 sec; normal, 33.5 sec; prothrombin time [PT], 12.8 sec; normal, 12.4 sec). Amantadine and intravenous ribavirin were started two weeks after the onset of illness. She progressed to respiratory failure and died with multiple organ failure one month after the onset of illness.

Patient 2 was a previously healthy 25-year-old Filipino female who presented with fever and respiratory symptoms for three days. On admission, she had clinical and radiological signs of left-lower-lobe consolidation. Initial investigations revealed leucopenia (WBC count, $2.29 \times 10^9/L$), lymphopenia (lymphocyte count, $0.28 \times 10^9/L$), thrombocytopenia (platelet count, $106 \times 10^9/L$) and prolonged clotting time (APTT, 40.7 sec; PT, 12.4 sec). Amantadine was started one week after the onset of illness. She progressed to respiratory failure and died with multiple organ failure one month after the onset of illness.

Virus detection

Virus isolation. Fresh autopsy tissues were homogenized and inoculated onto Madin-Darby canine kidney (MDCK) cells and observed daily for cytopathic effects. After 10 days of incubation, the cells were stained with influenza A-specific monoclonal antibodies (DAKO Imagen™, Cambridgeshire, UK) using the immunofluorescence technique.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were extracted from fresh-frozen tissues. Influenza A H5N1-specific sequences were detected by RT-PCR specific for the hemagglutinin gene of H5N1 as previously described [Yuen et al., 1998].

Immunohistochemistry. The presence of influenza A H5 antigens in tissues was detected by H5-specific monoclonal antibodies (364/1 and CP62 kindly provided by R.G. Webster, St. Jude's Children's Hospital, Memphis, Tennessee, USA) using the immunoperoxidase technique. The procedures were optimized using formalin-fixed and paraffin-embedded H5N1-infected MDCK cells. All immunostaining procedures were carried out in parallel with a positive control (H5-infected MDCK cells) and a negative control (the next serial tissue section with the omission of primary antibodies).

Serum cytokine measurement

Serum levels of interferon- γ (IFN- γ), soluble interleukin-2 receptor (sIL-2r), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) were determined by an enzyme immunoassay (Genzyme, MA). A pool of samples from patients admitted to hospital was included to verify the normal ranges provided.

RESULTS

Histologic findings

Patient 1

Hemato-lymphoid system. The bone marrow was markedly hypoplastic with reversed myeloid to erythroid ratio. Histiocytic hyperplasia with diffuse infiltration by reactive histiocytes exhibiting active hemophagocytosis was observed (Figure 1 A, B). Prominent iron deposition in the histiocytes was also evident. The lymph nodes sampled from various sites showed expansion of sinus spaces accompanied with prominent hemophagocytosis and focal necrosis (Fig. 1 C, D). The spleen was mildly enlarged with white pulp atrophy. The red pulps were expanded by reactive hemophagocytic histiocytes.

Other major organs. Macroscopically, both lungs were firm and consolidated with extensive hemorrhage and focal cystic changes. Histologic sections revealed organizing diffuse alveolar damage, interstitial fibrosis and cystically dilated air spaces. The pneumocytes exhibited reactive changes, but no viral inclusion was observed. Interstitial lympho-plasmacytic infiltration and scattered histiocytes with reactive hemophagocytic activity were occasionally observed. No evidences of superimposed infection were seen. A 3-cm focus of sub-capsular necrosis was observed in the liver. Extensive central lobular necrosis, mild fatty changes and activated Kupffer's cells with occasional hemophagocytic activity were detected in liver sections. Extensive acute tubular necrosis was observed in the swollen and congested kidneys. The brain was mildly oedematous and hemophagocytic histiocytes were found over the meninges. Multiple microscopic lesions consisting demyelinated areas and reactive histiocytes were found in the white matter of the cerebrum. No encephalitis or ventriculitis was seen.

Patient 2

Hemato-lymphoid system. The hypercellular bone marrow showed active hematopoiesis with left shift of the myeloid series, and histiocytes showed reactive hemophagocytosis. Reactive hemophagocytic activity, extra-medullary hematopoiesis and focal necrosis were observed in lymph nodes. The spleen was mildly enlarged and exhibited prominent extra-medullary hematopoiesis, reactive hemophagocytosis and expansion of red pulp (Fig. 1 E, F).

Other major organs. The pathologic features observed in the lungs were similar to that of patient 1 and without evidence of superimposed infection. The

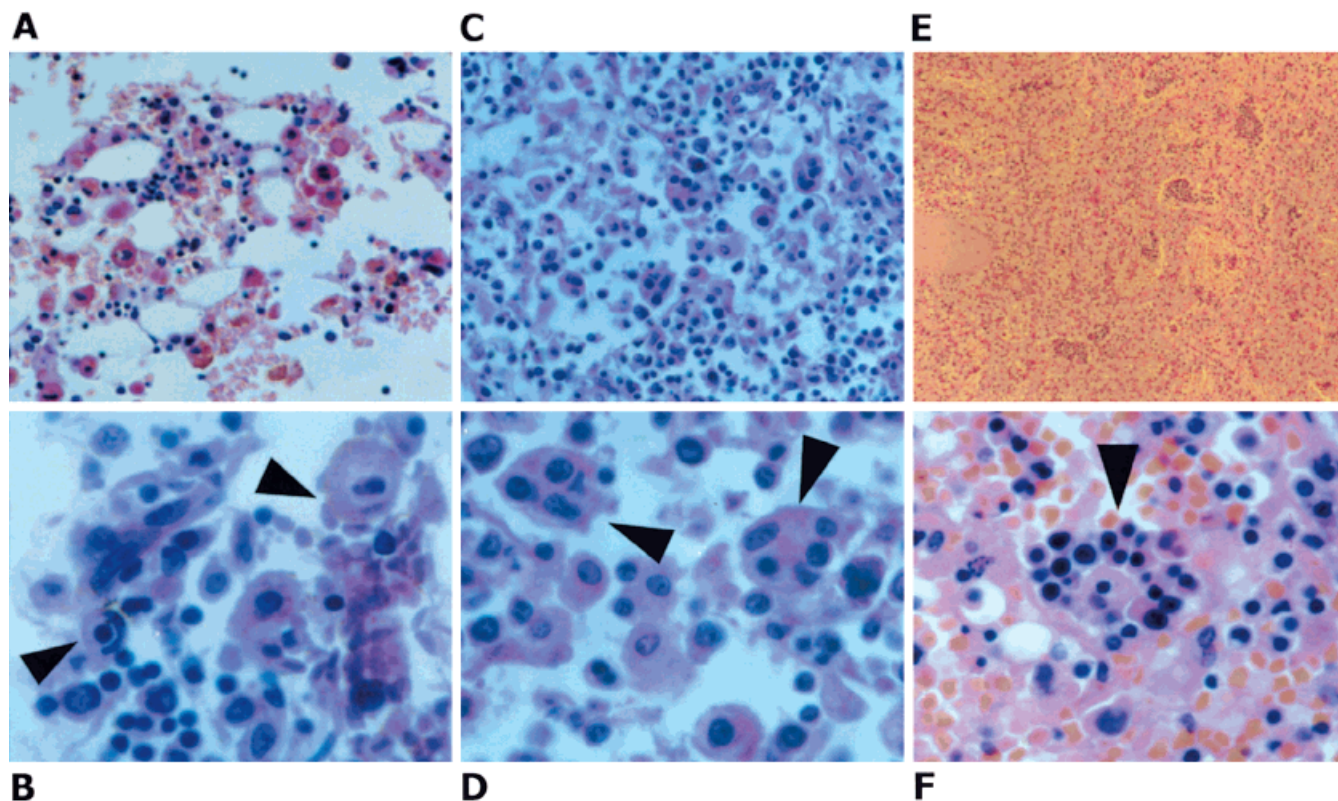


Fig. 1. **A** ($\times 100$) and **B** ($\times 400$): Bone marrow sections of patient 1 showing a hypoplastic marrow with histiocytic hyperplasia and hemophagocytic activity (hematoxylin and eosin); **C** ($\times 200$) and **D** ($\times 400$): Lymph node sections of patient 2 showing histiocytic proliferation in lymph node sinus with hemophagocytic activity and

ingestion of lymphocytes (hematoxylin and eosin); **E** ($\times 40$) and **F** ($\times 400$): Spleen sections of patient 2 showing expansion of red pulp and hemophagocytic histiocytes (hematoxylin and eosin). Arrow heads: histiocytes showing hemophagocytosis.

liver exhibited central lobular necrosis, micro- and macro-vesicular fatty changes and extra-medullary hematopoiesis. The Kupffer's cells appeared active with occasional hemophagocytic activity. Prominent acute tubular necrosis was observed in the congested and swollen kidneys. The brain was mildly swollen, otherwise no significant pathology was noted.

Since Epstein-Barr virus is known to be a major cause of reactive hemophagocytic syndrome, *in situ* hybridization for Epstein-Barr virus-encoded RNAs (EBERs) was undertaken for tissues showing features of hemophagocytosis and were all negative.

Virus detection

Influenza A H5N1 was not detected in any of the tissues examined, including the lungs, heart, liver, kidney, spleen, multiple lymph nodes, intestine, brain and bone marrow.

Cytokine measurement

Serum samples of patient 1 taken at day eight and day 11, and patient 2 taken at day seven after the onset of illness were available for cytokine measurement. The sIL-2r and IL-6 levels were elevated in all samples. IFN- γ were elevated in both early samples and returned to normal in the second sample of patient 1.

TNF- α was elevated in the second sample of patient 1 but not in the early samples of both patients (Table I).

DISCUSSION

The 1997 epidemic in Hong Kong was the first documentation of human disease associated with influenza A H5N1 infection. The explanation for the dramatic difference in clinical outcome, ranging from mild upper respiratory illnesses to multiple organ failure, is currently unclear [Yuen et al., 1998]. Most of the severely affected patients had hematologic manifestations, in particular lymphopenia and varying degrees of pancytopenia, impaired liver function and abnormal clotting profiles that had occurred early in the course of illness before the development of multiple organ failure. These presentations are in agreement with reactive hemophagocytic syndrome which was found to be the most prominent pathologic feature observed in both patients examined. Reactive hemophagocytic syndrome was described first in 1979 as a distinct clinico-pathologic entity [Risdaal et al., 1979], characterized by proliferation of benign hemophagocytic histiocytes in hematopoietic organs with frequent involvement of other organ systems. The clinical manifestations include varying degrees of peripheral blood cytopenia, frequently pancytopenia, coagulative

TABLE I. Cytokine Profile of Two Fatal Cases of Influenza A H5N1 Infection

Days after onset of illness	IF- γ (U/mL)	TNF- α (pg/mL)	sIL-2r (pg/mL)	IL-6 (pg/mL)
Patient 1				
Day 8	7.6*	6.6	4270*	78*
Day 11	0.7	26.5*	4932*	138*
Patient 2				
Day 7	14.4*	8.8	3005*	> 300*

*Cytokine levels above normal ranges (IF- γ , 0.97 +/- 0.51 U/mL; TNF- α , 9.13 +/- 7.23 pg/mL; sIL-2r, 2205 +/- 622 pg/mL; IL-6, 13 +/- 4 pg/mL).

abnormalities, impaired liver function and usually results in death [Henter et al., 1991]. Underlying immunologic defects or malignancies are known risk factors for reactive hemophagocytic syndrome. In both the immunocompetent and immunocompromised individuals, the syndrome is usually triggered by viral or bacterial infections [Favara, 1992], with Epstein-Barr virus (EBV) being the most common culprit [Wilson et al., 1981; Reisman and Greco, 1984]. In our patients, the possibility of EBV-associated reactive hemophagocytic syndrome was excluded by the negative results of EBERS detection. Influenza-associated reactive hemophagocytic syndrome is extremely rare, and has only been reported in a cluster of three children with acute leukemia [Potter et al., 1991]. Our cases represented the first documentation of influenza A, in particular H5N1, associated with reactive hemophagocytic syndrome in previously healthy individuals.

The pathogenesis of reactive hemophagocytic syndrome is still obscure. A model which is accepted generally suggests it to be a cytokine-driven condition triggered by various causes [Akashi et al., 1994; Imashuku et al., 1996]. The serum cytokine profiles observed in our patients are in keeping with this suggestion. In patient 1, IFN- γ was raised transiently early in the course of illness (day 8) and returned to a normal level at day 11, followed by an elevation of TNF- α . Although, the TNF- α level of patient 2 was within normal limits at day 7, owing to the lack of a later sample, it was not possible to determine whether elevation of TNF- α had also increased at a later stage as observed in patient 1. Elevation of sIL-2r and IL-6 was observed in all samples studied, which may indicate a sustained release of both cytokines. Elevation of sIL-2r, IL-6 and TNF- α has been associated with poor prognosis in patients with reactive hemophagocytic syndrome. The phagocytic activity and systemic manifestations of the syndrome are also believed to be mediated by these cytokines [Akashi et al., 1994; Imashuku et al., 1996]. It has been shown that avian influenza A viruses can infect macrophages resulting in a marked elevation of mRNA of IL-1, IL-6 and TNF- α [Peschke et al., 1993; Hofmann et al., 1997; Lehmann et al., 1996], but the precise mechanism of H5N1 infection leading to the hypercytokinemia stage in humans is still unclear.

In view of the early onset of multiple organ failure, attempts were made to detect H5N1 from multiple

extra-pulmonary tissues where direct viral invasion might have contributed to organ malfunction. Although the results of virus detection were all negative, viral tropism to extra-pulmonary tissues cannot be excluded, since in both patients, respiratory specimens became negative for the virus soon after the initiation of antiviral treatment. In addition, both patients died at one month after the onset of illness, making the interpretation of virus detection on post-mortem tissues difficult. An attempt was also made to detect viral antigens from two other patients who died relatively earlier. Immunostaining was performed on the post-mortem lung and renal tissues taken from a 54-year-old male who died of H5N1 at day 11 after the onset of illness, and from the para-mortem liver tissue of a 3-year-old boy who died at day 10 after the onset of illness. In these two patients, a full post-mortem was not performed and these tissues were the only available specimens. Influenza A H5-specific antigens were detected in the lung tissues only. In addition, neither viral inclusions nor inflammatory responses that might associate with viral invasion were observed from the renal and liver tissues. Although the influenza A H5N1 strain isolated from the first identified case (3-year-old boy) was shown to be pantropic in chickens [Subbarao et al., 1998], based on the available information, evidence of direct viral invasion of influenza A H5N1 in extra-pulmonary tissues in humans is still lacking. Further studies are required to elucidate this area.

It is difficult to draw a definitive conclusion on the pathogenesis based on the limited number of patients examined. Nevertheless, based on the available findings, it is postulated that a patient with severe influenza A H5N1 infection might progress through a pathogenic pathway distinct from that of the usual human subtypes H1-H3. Replication of avian influenza A H5N1 in the human respiratory tract might have a potential to trigger a stage of hypercytokinemia and complicated with hemophagocytic syndrome. Although elevation of cytokines can also occur in the usual influenza A infections, these infections do not progress to reactive hemophagocytic syndrome. The hematologic manifestations and the subsequent multiple organ failure might also be partly due to hemophagocytic syndrome. However, due to insufficient material, it is difficult to determine the role of direct lytic viral infection in causing lymphopenia and multiple organ

failure. Further studies on the tropism and pathogenesis of this potentially fatal human pathogen are crucial to optimize the therapeutic strategy.

ACKNOWLEDGMENT

We thank R.G. Webster, St Jude's Children's Hospital, Memphis, Tennessee, USA, for providing the monoclonal antibodies specific for the H5 hemagglutinin of influenza A.

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