**Short Communication**

H5N1-Infected Cells in Lung with Diffuse Alveolar Damage in Exudative Phase from a Fatal Case in Vietnam

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**SUMMARY:** Necropsied lung tissues of three fatal cases with avian influenza A virus (H5N1) infection in Vietnam were analyzed to detect H5N1 virus-infected cells. Formalin-fixed and paraffin-embedded lung tissue sections showed typical histological features of diffuse alveolar damage (DAD) in all cases. Immunohistochemistry for the influenza A virus nucleoprotein antigen revealed positive signals of bronchiolar and alveolar epithelial cells in only one patient, who exhibited DAD with an exudative phase and died on the 6th day after onset. However, no signal was detected in the other two cases of DAD with a proliferative phase. These patients died on day 16 and day 17 after onset, respectively. H5N1 virus antigens were detected predominantly in epithelial cells in terminal bronchioles and in alveoli, i.e., type I and type II alveolar pneumocytes, and in alveolar macrophages. The pathogenesis of exudative DAD caused by H5N1 infection is discussed.

Highly pathogenic avian influenza A H5N1 virus (H5N1) infection has been reported to cause severe respiratory disease. In 1997, H5N1 was first isolated in Hong Kong from tracheal aspirates of a 3-year-old boy with a fatal respiratory illness (1-3). In 2003, human disease associated with H5N1 re-emerged (4). Since then, the number of confirmed fatal human H5N1-infected cases has increased and now totals approximately 200 cases. These cases occurred, predominantly, in Vietnam, Thailand, and Indonesia (5-9). The histopathological data for H5N1 virus infection in humans were, however, limited (3,4,6,8,10-12), and the pathogenesis of the disease remains unclear. Examination of ex vivo infected lung tissues showed that influenza A virus nucleoprotein (InfA-NP) was detected in pneumocytes and in alveolar macrophages (13). Also the pattern of viral attachment in human respiratory tract sections showed that H5N1 attached to the apical cell membrane of bronchiolar cells, type II pneumocytes and alveolar macrophages (14,15). The postmortem study of H5N1-infected patients has recently been published for the first time (16).

In the present study, we describe the histopathological findings from three fatal cases of H5N1 infection from the National Hospital of Pediatrics in Hanoi, Vietnam. The detailed clinical findings of Case 1 and Case 2 have been described previously (5). On admission, all patients presented with fever, cough, and dyspnea, and H5N1 virus was detected in tracheal fluids by reverse-transcriptase polymerase chain reaction (RT-PCR) before death occurred. The duration of the disease in Case 1, 6 days, was much shorter than in the other two cases (Table 1). Small pieces of lung tissues in the lower respiratory tract were necropsied and histological and immunohistochemical examinations were carried out on formalin-fixed and paraffin-embedded lung tissues.

The hematoxylin and eosin-stained lung sections of Case 1 demonstrated typical histological features of diffuse alveolar damage (DAD) with an exudative phase (Fig. 1a). Eosinophilic hyaline membrane was found on alveolar ducts and on alveoli. The alveolar space was filled with proteinaceous exudates containing erythrocytes, macrophages, and cell debris. The alveolar septa were thickened by edema with mild inflammatory infiltration, consisting of lymphocytes and macrophages. In Cases 2 and 3, hyaline membrane formation was focally found, and the proliferation of fibroblasts in the interstitial space was marked in comparison to Case 1. Mild interstitial inflammation and proliferation of type II pneumocytes with bizarre and cuboidal features were observed (Fig. 1c), indicating that Cases 2 and 3 were in the proliferative (repair) phase of DAD. Squamous cell metaplasia in the bronchiolar epithelium was also observed (Fig. 1d). Focal accumulation of neutrophils in the alveolar space was found in Case 3, suggesting pulmonary bacterial infection. These histological features were similar to those reported previously in fatal human H5N1 influenza A virus-infected cases (4,8,10,11).

To detect the influenza A virus antigen, the sections were immunostained with an avidin-biotin complex immunoperoxidase method (LSAB kit/HRP/DAB; Dako Cytomation, Copenhagen, Denmark) using a mouse monoclonal antibody against InfA-NP (17). Positive signals for InfA-NP were detected in 6 of 6 blocks of lung tissue from Case 1, whereas they were not found in those from Case 2 or 3. The signals were found mainly in alveolar epithelial cells and in interstitial cells (Fig. 1b). The many positive cells were interpreted as type II pneumocytes and/or alveolar macrophages, but the positive cell presented in the inset in Fig. 1b was considered to be a type I pneumocyte based on its histological location and morphology. H5N1-RNA was also detected by real-time RT-
PCR in paraffin-embedded lung sections from Case 1 only (18). In DAD with a proliferative phase, as in Cases 2 and 3, viral antigens and nucleic acids were not detected.

To characterize virus-infected cells, confocal laser scanning microscopy was used to visualize double immunofluorescence staining for InfA-NP and for cell-type specific marker proteins of epithelial cells, macrophages, and endothelial cells. The antibodies used are shown in Table 2. Alexa Fluor 568-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, Oreg., USA) and Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes) were used as secondary antibodies. InfA-NP signals were detected most

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**Table 1.** Histopathological findings in the lung of H5N1 fatal cases in Vietnam

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Days from onset to death</th>
<th>Histology in lung sections</th>
<th>RT-PCR for H5N1 (tracheal fluids)</th>
<th>RT-PCR for H5N1 (paraffin-embedded sections of lung)</th>
<th>Immunohistochemistry for InfA-NP antigen and co-localization with cell marker proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^1)</td>
<td>12/F</td>
<td>6</td>
<td>DAD with an exudative phase, Hyaline membrane formation</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive for InfA-NP antigen, and colocalized with AE1/AE3, EMA, SPA, SPD, CD68, CD34</td>
</tr>
<tr>
<td>2(^2)</td>
<td>5/M</td>
<td>17</td>
<td>DAD with a proliferative (repair) phase</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative for InfA-NP antigen</td>
</tr>
<tr>
<td>3</td>
<td>4/M</td>
<td>16</td>
<td>DAD with a proliferative (repair) phase Microabscesses</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative for InfA-NP antigen</td>
</tr>
</tbody>
</table>

\(^1\): Patient 1 in Ref (5).
\(^2\): Patient 2 in Ref (5).

M, male; F, female; DAD, diffuse alveolar damage; InfA-NP, influenza virus A nucleoprotein; EMA, epithelial membrane antigen; SPA, surfactant protein A; SPD, surfactant protein D.

**Table 2.** Antibodies used for double immunofluorescence staining

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody type</th>
<th>Stained cells</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytokeratin (AE1/AE3)</td>
<td>mouse monoclonal</td>
<td>epithelial cell of bronchiole</td>
<td>Dako</td>
</tr>
<tr>
<td>epithelial membrane antigen (EMA)</td>
<td>mouse monoclonal</td>
<td>epithelial cell</td>
<td>Dako</td>
</tr>
<tr>
<td>surfactant apoprotein A (SPA)</td>
<td>mouse monoclonal</td>
<td>type II alveolar pneumocyte</td>
<td>Dako</td>
</tr>
<tr>
<td>surfactant apoprotein D (SPD)</td>
<td>rabbit polyclonal</td>
<td>type II alveolar pneumocyte</td>
<td>Chemicon(^1)</td>
</tr>
<tr>
<td>CD68 (KP1)</td>
<td>mouse monoclonal</td>
<td>alveolar macrophage</td>
<td>Dako</td>
</tr>
<tr>
<td>CD68 (PG-M1)</td>
<td>mouse monoclonal</td>
<td>alveolar macrophage</td>
<td>Dako</td>
</tr>
<tr>
<td>CD34</td>
<td>mouse monoclonal</td>
<td>endothelial cell</td>
<td>Immunotech(^2)</td>
</tr>
<tr>
<td>influenza A virus nucleoprotein</td>
<td>mouse monoclonal</td>
<td>influenza A virus infected cell</td>
<td>in-house Ref. (17)</td>
</tr>
<tr>
<td>influenza A virus nucleoprotein</td>
<td>mouse monoclonal</td>
<td>influenza A virus infected cell</td>
<td>in-house Ref. (17)</td>
</tr>
</tbody>
</table>

\(^1\): Chemicon, Temecula, Calif., USA.
\(^2\): Immunotech, Marseille, France.

**Fig. 1.** Hematoxylin and eosin stainings and immunohistochemistry for influenza virus A nucleoprotein (InfA-NP) in Case 1. (a) Hyaline membrane formation is observed on the alveolar walls. In the interstitial space, edema and mild inflammatory cell infiltrates are observed (Case 1). (b) InfA-NP antigens are detected in alveolar epithelial cells and in the interstitial space. InfA-NP-positive, type I pneumocyte is indicated in the inset. (c) Mild interstitial inflammation and proliferation of type II pneumocytes with bizarre and cuboidal features were observed (Case 3). (d) Squamous cell metaplasia in the bronchiolar epithelium was also observed (Case 2). Scale bar = 100 μm.
frequently in epithelial (EMA-positive) cells. They were also detected in AE1/AE3, SPD, SPA, and CD68-positive cells (Fig. 2), indicating that H5N1 virus antigens were present predominantly in the epithelial cells in terminal bronchioles and alveoli, mainly in type II alveolar pneumocytes and in alveolar macrophages. A few H5N1 virus-infected type I pneumocytes were also suggested by double-positive staining for InfA-NP and for EMA, in combination with distinctive morphology. Although the number was very few, the InfA-NP signal was also detected in CD34-positive cells, suggesting that the H5N1 had infected some CD34-positive endothelial cells. Further investigation will be necessary to confirm the H5N1 infection of human endothelial cells, as has been observed in the endothelial cells of chickens and other birds (19). The localization of InfA-NP antigen within the cell was determined by counterstaining with TO-PRO-3 nucleic acid staining (Molecular Probes). Some InfA-NP signals were detected in nuclei (Fig. 3a) and others were detected in the cytoplasm (Fig. 3b). Histologically, in the early phase of infection, InfA-NP antigen was localized in the nucleus, while in the late phase of infection, InfA-NP antigen was localized in the cytoplasm (20). These observations suggested that viruses were in the proliferative stage in the early phase of H5N1 infection. The histopathological data

Fig. 2. The phenotype of influenza virus A nucleoprotein (InfA-NP) positive cells. InfA-NP immunoreactivity (a, d, h, l, p, t) (red color) and cytokeratin (b), EMA (e), SPD (i), CD68 (Kp1) (m), CD68 (PGM-1) (q) or CD34 (u) immunoreactivity (green color). Co-localization is presented respectively (c, f, j, n, r, v). Differential interference contrast (DIC) images are also shown (g, k, o, s, w). Original magnifications, ×400.

Fig. 3. Immunofluorescence staining of InfA-NP antigen in infected epithelial cells. InfA-NP immunoreactivity (red color), TO-PRO-3 nucleic acid staining (blue color) and merged images (pink color) are shown. Some were analyzed with differential interference contrast (DIC) images. The InfA-NP antigen was localized in nuclei (a) or in cytoplasm (b). Original magnifications, ×400.
are summarized in Table 1.

Avian influenza viruses have been found to preferentially bind to sialic acid-α-2,3-Gal (SAα2-3)-linked oligosaccharides, while human influenza viruses were found to bind to SAα2-6-linked oligosaccharides (21), although these findings were made in vitro or ex vivo experiments. As an in vivo examination, we performed an analysis with the double-staining technique using a monoclonal antibody against InfA-NP in combination with either biotinylated Maackia amurensis agglutinin (MAA) lectin (Vector Laboratories, Burlingame, Calif., USA) which is specific for SAα2-3-linked oligosaccharides, or with Sambucus nigra agglutinin (SNA) lectin (EY Laboratories, San Mateo, Calif., USA) which is specific for SAα2-6-linked oligosaccharides. In the alveoli, many cells were not stained by SNA lectin but were stained by MAA lectin, suggesting that they express SA α2-3-linked oligosaccharides, as found in previous reports (21). Unexpectedly, the InfA-NP-positive cells were not double-stained by MAA lectin.

Although the materials were restricted to small pieces of lung tissue in the lower respiratory tract, the evidence in the present study showed that several types of cells in the lung, namely type I and type II alveolar pneumocytes, epithelial cells in terminal bronchioles, macrophages in the alveolar space and CD34-positive endothelial cells in the interstitial tissues, were involved in the disease. The evidence in Case 1, the case with H5N1 infection who died on day 6 after onset, strongly suggests that H5N1 may infect the epithelial cells of alveolar tissues in the early clinical phase and can thereafter be transmitted to adjacent cells. The dissemination of infection among these cells was supposed to be accompanied by the release of pro-inflammatory cytokines from the infected alveolar macrophages (4, 10, 12), resulting in rapid progression from DAD with an exudative phase to that with a proliferative phase.

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REFERENCES


