Immunohistochemical and In Situ Hybridization Studies of
Influenza A Virus Infection in Human Lungs

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Abstract

Influenza viruses are important human pathogens of the respiratory tract that cause substantial morbidity and mortality almost every year.¹ Influenza viruses may circulate sporadically, during local outbreaks as part of a widespread regional or national epidemic, or as a worldwide epidemic (pandemic).² During epidemics, the highest mortality has been documented among the very young and very old and among adults with chronic cardiopulmonary disease.³ In the 20th century, influenza pandemics occurred in 1918, 1957, and 1968 and accounted for more than 600,000 excess deaths in the United States alone.⁴ Much of our understanding of the pathology of influenza infection in humans comes from studies of these pandemics.⁵⁻¹⁰ The classic pathologic descriptions include early changes, such as necrotizing bronchiolitis, vessel thrombosis, interstitial inflammation, and hyaline membrane formation, accompanied by varying degrees of intra-alveolar edema, hemorrhage, and inflammation.⁵⁻¹⁰ As the disease progresses, diffuse alveolar damage with fibrosis and squamous metaplasia occurs. Secondary bacterial pneumonia may occur as a complication of influenza virus infection,²,⁹ and, in autopsy material, it often is difficult to distinguish the pathologic changes attributable to viral infection from those resulting from secondary bacterial infection.⁶

Influenza viruses are single-stranded RNA viruses that belong to the orthomyxovirus family.¹¹ The viral envelope contains 2 surface glycoproteins: the hemagglutinin (HA), which is responsible for attachment and penetration of the virus into cells, and the neuraminidase, which is responsible for release of progeny virions from the infected cells. Within the envelope are a matrix protein (M), which encloses the viral RNA associated with the nucleocapsid protein (NP), and 3 polymerase proteins responsible for replication. Influenza viruses are divided into 3 types (A, B,
and C) based on antigenicity of the NP and are subtyped based on the antigenicity of the HA and neuraminidase proteins. In the 20 century, the H1, H2, and H3 hemagglutinin subtypes, in combination with N1 or N2 neuraminidase subtypes, caused widespread epidemics and pandemics among humans.

The present report describes the development of immunohistochemical and in situ hybridization (ISH) assays for the detection of influenza A virus antigen and nucleic acid in infected cells. The diagnostic usefulness of both techniques was evaluated in formalin-fixed paraffin-embedded tissues from 6 fatal cases with culture-proven influenza A virus infection and from 2 cases in which influenza virus infection was suspected on the basis of clinical symptoms but from which no isolate was obtained. The results demonstrate the successful application of these techniques and describe the distribution of viral antigens and nucleic acids in human tissues.

Materials and Methods

Patient Tissues

Formalin-fixed paraffin-embedded lung tissue blocks were available from 8 patients’ autopsies. In 6 patients, influenza A virus had been cultured from the nasopharynx or lung; in 2 cases, influenza infection was suspected clinically, but no virus specimen had been obtained. Other tissues available for study included blocks from the central nervous system in a case with concomitant encephalitis and from the heart in a case with myocarditis. Routine H&E-stained sections were examined in all cases.

Immunohistochemical Assay

For the immunohistochemical assay, 3-µm tissue sections were deparaffinized and rehydrated through a series of graded alcohol solutions. Sections were placed in the DAKO autostainer (DAKO, Carpinteria, CA), digested in 0.1 mg/mL of Proteinase K (Boehringer-Mannheim, Indianapolis, IN) in a 0.6-mol/L concentration of tris(hydroxymethyl)aminomethane (Tris) (pH 7.5)—0.1% calcium chloride (Proteinase K buffer) for 15 minutes, and later blocked with methyl)aminomethane (Tris) (pH 7.5)–0.1% calcium chloride in a 0.6-mol/L concentration of tris(hydroxymethyl)aminomethane. The sections were denatured in 70% formamide with 2× standard saline citrate (1× standard saline citrate is a 0.15-mol/L concentration of sodium chloride plus a 0.015-mol/L concentration of sodium citrate) for 10 minutes at 70°C and later placed in ice-cold 70% ethanol for 10 minutes, followed by dehydration in graded alcohol solutions. The slides were air dried and then prehybridized for 30 minutes at room temperature in hybridization buffer (Boehringer-Mannheim) in 50% formamide. Two RNA probes that hybridized to HA and NP genes were developed. The HA RNA probe was generated from a full-length complementary DNA (cDNA) clone of the H3 HA from influenza A/Beijing/4/89 virus positioned in plasmid pUC19 behind a T7 RNA polymerase promoter to generate a negative (vRNA)-sense transcript for the detection of H3 HA messenger RNA (mRNA; Influenza Branch, Centers for Disease Control and Prevention, Atlanta, GA). A digoxigenin-labeled RNA probe of approximately 450 nucleotides was generated by runoff transcription of purified plasmid cDNA linearized with restriction endonuclease XhoI according to the manufacturer’s instructions (Boehringer-Mannheim). The NP RNA probe was generated from a full-length cDNA clone of the NP from influenza A/Leningrad/134/57 (H2N2) virus positioned in plasmid pCR behind a T7 RNA polymerase promoter to generate a negative (vRNA)-sense transcript for the detection of NP mRNA. A digoxigenin-labeled probe of approximately 420 nucleotides was generated by runoff transcription of purified cDNA linearized with restriction endonuclease HindIII.

The probes were applied to the tissue sections, covered with parafilm (Fisher Scientific), and hybridized overnight.
at 37°C in a humidified chamber. Parafilm covers were removed, and sections were washed in 50% formamide, 2× standard saline citrate at 44°C, and blocked in 20% normal sheep serum. Mouse antidigoxigenin was applied to the sections and detected by serial application of biotinylated immunoglobulins, streptavidin-alkaline phosphatase, and naphthol–fast red substrate as described for the immunohistochemical assay in the DAKO autostainer. Sections were counterstained in Mayer hematoxylin.

Optimization of the hybridization assay with both probes was performed on formalin-fixed paraffin-embedded influenza A virus–infected MDCK cells. The specificity of both influenza probes was confirmed using a digoxigenin-labeled enterovirus RNA probe as a negative control probe.

Results

Table 1 gives pertinent clinical information for the 8 patients studied. Two patients (cases 2 and 6) were infected during community outbreaks of influenza. One patient (case 2), an elderly male resident of a nursing home where an influenza outbreak had occurred, developed respiratory symptoms that were treated with amantadine a few hours before his death. One patient (case 6) died during a summer outbreak of influenza A virus infections in persons who traveled to Alaska on cruise ships.14 Four of the patients in the present study had conditions predisposing them to complications from influenza infection that ranged from hay fever to severe immunodeficiency (AIDS). The most frequent symptom in this group of patients was fever. In 7 cases, the time from onset of symptoms to death was 1 week or less.

Influenza A virus was cultured from samples obtained during autopsy of cases 1, 2, 3, 7, and 8. In cases 4 and 5, no culture specimen was obtained. In case 6, a positive culture isolate was obtained early during hospitalization. Patient 8 shed influenza A virus in respiratory secretions for 8 months before death.

The patients can be divided into 2 groups according to the predominant histopathologic features and immunohistochemical results: the tracheobronchitis group had bronchitis and positive immunohistochemical staining results, and the alveolitis group had alveolitis and negative immunohistochemical staining results. Table 2 shows the histopathologic group and immunohistochemical and ISH findings in lung tissue sections in each case.

Five patients (cases 1-5) had bronchitis as the most prominent pathologic feature. The bronchi showed varying degrees of lymphohistiocytic inflammatory infiltrate. The bronchial epithelial lining showed 1 of 3 characteristics: normal epithelium, loss of cilia and mucus depletion on cells still attached to the basement membrane, or partial areas of cell detachment. The bronchial lumen had abundant necrotic material mixed with inflammatory cells and detached necrotic epithelial cells. The first 3 cases had varying degrees of interstitial inflammatory infiltrates in the areas surrounding the larger bronchi with necrotizing bronchitis; the infiltrate was composed mostly of mononuclear cells with some neutrophils. Other pathologic features found in the lung tissue of these patients included peripheral lung fibrosis and emphysematous changes in case 2, hypertrophy of the peribronchial muscle consistent with asthma in case 3, and multiple small abscesses in case 4.

The second group of patients (cases 6-8) had alveolitis. The alveolitis consisted of abundant lymphohistiocytic infiltrate in the alveoli extending into the surrounding interstitium. The alveolitis tended to be around bronchi that showed inflammatory infiltrate and partial destruction of the epithelium. Other pathologic features found in the lung tissue of these patients included peripheral lung fibrosis and several caseating granulomas that contained acid-fast bacilli in case 6 and acute bacterial pneumonia and intra-alveolar hemorrhage in case 7.
The other relevant nonpulmonary pathologic features found in these cases were encephalitis in case 1, lymphocytic myocarditis in case 3, and a brain abscess due to *Toxoplasma* species in case 8.

Positive immunohistochemical results were seen in the 5 patients in whom bronchitis was the main feature. In these cases, staining was observed only on a proportion of the blocks studied. Staining was focal in the bronchial epithelial cells, whether they were intact covering the bronchi or detached in the necrotic material [Image 1]. In cases 1 and 2, staining also was present in mononuclear inflammatory cells in the areas with pneumonitis (Image 1) [Image 2]. The anti-NP antibody primarily stained the cell nucleus, although faint granular staining also was noted in the cytoplasm. The anti-HA antibody stained mainly the cytoplasm (Images 1 and 2). Influenza viral antigens were not detected in sections of the central nervous system (case 1) or heart (case 3) by using immunohistochemistry.

### Table 2

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Histopathologic Group</th>
<th>Immunohistochemistry</th>
<th>In Situ Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tracheobronchitis</td>
<td>Positive in bronchial epithelium, rare interstitial macrophages and endothelium (4/4)</td>
<td>Positive in bronchial epithelium (less abundant than in immunohistochemical testing) and in interstitial cells (3/4)</td>
</tr>
<tr>
<td>2</td>
<td>Tracheobronchitis</td>
<td>Positive in bronchial epithelium, and rare interstitial macrophages (7/15)</td>
<td>Negative (0/15)</td>
</tr>
<tr>
<td>3</td>
<td>Tracheobronchitis</td>
<td>Positive in bronchial epithelium (2/15)</td>
<td>Positive in bronchial epithelium (less abundant than in immunohistochemical testing) (2/15)</td>
</tr>
<tr>
<td>4</td>
<td>Tracheobronchitis</td>
<td>Positive in bronchial epithelium (2/2)</td>
<td>Positive in bronchial epithelium (less abundant than in immunohistochemical testing) (2/2)</td>
</tr>
<tr>
<td>5</td>
<td>Tracheobronchitis</td>
<td>Positive in bronchial epithelium (3/3)</td>
<td>Positive in bronchial epithelium (rare, scattered cells) (2/3)</td>
</tr>
<tr>
<td>6</td>
<td>Alveolitis</td>
<td>Negative (0/4)</td>
<td>Negative (0/4)</td>
</tr>
<tr>
<td>7</td>
<td>Alveolitis</td>
<td>Negative (0/10)</td>
<td>Negative (0/10)</td>
</tr>
<tr>
<td>8</td>
<td>Alveolitis</td>
<td>Negative (0/3)</td>
<td>Negative (0/3)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are number of slides positive/number of slides studied.
ISH-positive staining was present in 4 of 5 patients with bronchitis (cases 1 and 3-5). Staining was seen in the same areas as with the immunohistochemical assay but in less abundance. The NP and HA probes hybridized in both the cell nucleus and cytoplasm.

The results of influenza A immunohistochemical and ISH assays performed on control cells and immunohistochemical assays performed on cases with lung and heart disease due to other infectious agents were negative. In case 5, an immunohistochemical assay using an antibody against Streptococcus pneumoniae demonstrated clusters of cocci in the abscesses and bronchi.

Discussion

In the cases studied, we found a histopathologic spectrum that included patients with prominent bronchitis and mild interstitial inflammatory infiltrates and patients with a predominant alveolitis. Bronchoscopy studies of patients with acute, nonfatal, influenza virus infection have demonstrated that the pathologic features are more prominent in larger bronchi than in the trachea and that the same patient can have varying degrees of inflammation from one bronchus to another. Walsh et al also found that the epithelial damage varied, with extensive desquamation of the epithelium noted in some areas, and columnar cells exhibiting vacuolization, edema, and absence of cilia in others. The changes described in patients with acute, nonfatal, influenza virus infection are very similar to the findings we describe in the patients with bronchitis. These changes correspond to what has been described as early infection.

By using immunohistochemical and ISH assays, we detected influenza A viral antigens and nucleic acids in intact and detached bronchial epithelium of the patients with bronchitis. The bronchial damage observed was extensive, and we were able to demonstrate infection in very few epithelial cells. Viral infection of epithelial cells is at least partially responsible for histologic features such as loss of cilia, eosinophilic cytoplasm, and eventual cell necrosis and detachment. However, the extensive histologic changes seen in patients with bronchitis, such as ulcers and necrosis, are due not only to viral infection of the epithelial cells, but also to the inflammatory host response.

In the present study, we were unable to detect viral antigens or nucleic acids in the 3 patients whose predominant histopathologic change was alveolitis. It is unclear why patients with alveolitis had a lack of immunohistochemical and ISH staining. Although the number of cases examined in the present series was small, it does not seem that length of illness could be the only factor causing the histopathologic changes. In addition, we can postulate that host factors may determine the histologic outcome; thus, influenza A virus infection could trigger an immune response causing alveolitis in some patients. This would be comparable to the mechanism suggested for myocarditis and encephalitis in which the virus is not detected in the heart or brain.

Secondary bacterial pneumonia is the most frequent complication (up to 75%) of fatal cases of influenza virus infection. Staphylococcus aureus and S pneumoniae are the most frequent organisms found in the secondary pneumonia. In general, by the time secondary bacterial pneumonia has occurred, it has been difficult to distinguish the pathologic changes due to influenza virus from those of secondary bacterial infections. By using immunohistochemical and ISH assays, we were able to detect necrotizing bronchitis with evidence of influenza A virus and multiple small abscesses with S pneumoniae in case 5.

Our study demonstrated immunohistochemical and ISH staining of mononuclear cells in the interstitium in 1 case. Presence of antigens in interstitial cells previously has been demonstrated by immunofluorescence assays and may represent phagocytosed viral antigens. Influenza viruses are negative single-strand RNA viruses with no DNA intermediates. The probes used in the present study were designed to detect virus-specific positive-strand mRNA that would be present only if the virus were replicating in the cells. The presence of actively replicating influenza A virus in mononuclear interstitial cells could explain the route by which the influenza virus gains access into the circulation, allowing detection in blood.
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and other nonpulmonary tissues. It also may explain, in part, the pathogenesis of interstitial pneumonitis, alveolitis, diffuse alveolar damage, myocarditis, and encephalitis that could result from a combination of inflammatory cytokines and other virally induced products.

Immunohistochemical and ISH assays have been used to detect influenza viral antigens and nucleic acids in animal models. Viral antigens were demonstrated in the bronchial epithelium, alveolar cells, vascular endothelium, neurons, and glial cells of mice that were inoculated intranasally with different strains of influenza virus. In human tissues, viral antigens were found only in the bronchial epithelium and interstitial macrophages of the lungs. We performed immunohistochemical and ISH tests on the brain (case 1) and the heart (case 3) and were not able to detect influenza antigens or nucleic acids. Possible explanations for the differences between human tissues and animal models are the fact that viral strains used in animal models are pantropic, that extrapulmonary organs of humans are more resistant to influenza virus infection, or that only a limited amount of human tissues have been studied. To evaluate the possibility of influenza virus infection in different human cell types, it will be necessary to analyze a wider array of tissues from culture-proven influenza cases by using immunohistochemical and ISH assays, as well as other techniques, such as polymerase chain reaction.

Clinical diagnosis of influenza virus is presumptive, and definitive diagnosis requires laboratory confirmation. Traditional diagnostic methods include viral isolation or serologic testing. In culture-proven cases, the histopathologic changes in the lung are nonspecific, and viral inclusions are not seen. By using immunohistochemical assays, we found viral antigens in formalin-fixed paraffin-embedded lung samples that contained larger bronchi with bronchitis, and none of the negative control cases showed staining. In the present study, we were able to find viral antigens in only 3 of 6 culture-confirmed cases; thus, a positive immunohistochemical result indicates infection, but a negative result does not rule out influenza A viral infection. On the other hand, immunohistochemistry proved to be helpful for confirming the diagnosis of influenza A virus infection in 2 of our cases in which infection was suspected but no cultures were performed and only formalin-fixed paraffin-embedded tissues were available. With ISH, we showed the presence of nucleic acids in formalin-fixed tissues in 4 cases, but the amount of cells stained and the intensity was lower than with immunohistochemical assays. Theoretically, these techniques, in particular the immunohistochemical assay, could be used to detect influenza virus in bronchoalveolar lavage specimens as is done with immunofluorescence assays. Advantages of using immunohistochemistry would include cytologic examination of the specimen and retaining a permanent record.

We developed immunohistochemical and ISH tests that can be performed on formalin-fixed paraffin-embedded tissues and can be useful as confirmatory diagnostic tests when fresh specimens are not available for culture. These techniques offer the advantage of localizing viral antigens and nucleic acids while preserving morphologic features, thus helping in our understanding of the extent of viral infection relative to the host inflammatory responses.

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References


