To determine if fatal infections caused by different highly virulent influenza A viruses share the same pathogenesis, we compared 2 different influenza A virus subtypes, H1N1 and H5N1. The subtypes, which had shown no pathogenicity in laboratory mice, were forced to evolve by serial passaging. Although both adapted viruses evoked diffuse alveolar damage and showed a similar 50% mouse lethal dose and the same peak lung concentration, each had a distinct pathologic signature and caused a different course of acute respiratory distress syndrome. In the absence of any virus labeling, a histologist could readily distinguish infections caused by these 2 viruses. The different histologic features described in this study here refute the hypothesis of a single, universal cytokine storm underlying all fatal influenza diseases. Research is thus crucially needed to identify sets of virulence markers and to examine whether treatment should be tailored to the influenza virus pathotype.

According to the World Health Organization, influenza annually infects 5%-15% of the global population, causing 3-5 million cases of severe illness and \( \approx 500,000 \) reported deaths. The persistence of influenza A virus (H5N1) in poultry populations over the past 6 years and the ability of those viruses to cause fatal infections in humans, along with the recent pandemic (H1N1) 2009 outbreaks, have raised fears of a renewed catastrophic influenza outbreak comparable to that of 1918, which caused death in 0.2%-8% of those infected in various countries and \( \approx 50 \) million deaths worldwide (1). Standard influenza symptoms include fever, cough, headache, sore throat, and dehydration, with some reports of diarrhea, vomiting, and bleeding from the mouth or throat. In benign cases, not all of these symptoms are exhibited. In severe cases, additional signs typical of either secondary bacterial pneumonia or acute respiratory distress syndrome (ARDS) occur. Notably, these 2 manifestations are those that cause death in patients with influenza, whether seasonal or pandemic or caused by the 1918 subtype H1N1 strain or by recent subtype H5N1 strains.

The catastrophic lethality of the 1918 pandemic makes it paramount that we understand the disease pathogenesis of both severe forms of influenza. Because most secondary bacterial pneumonias can be controlled with antimicrobial agents, prevention and treatment of influenza-associated ARDS are the major medical challenges that must be addressed to reduce the influenza-related death rate. This requires more knowledge about the pathogenesis of ARDS. Alterations in human and mouse lungs have been described for fatal virus infections with pandemic virus strains (subtypes H1N1, H2N2, and H3N2 strains of 1918, 1957, and 1968, respectively) or subtype H5N1 strains. They are all characterized by similar lung dysfunctions and lesions (2,3). The lung becomes flooded as its alveolocapillary membranes leak, and the alveoli fill with body fluids. Consequently, the exchange of carbon dioxide and oxygen is reduced, and fatal acute lung failure ensues. The histologic findings depend on the stage of the disease. Edema, epithelial necrosis, fibrin, and hyaline membranes are found during the early exudative phase, and fibroblast and type II cell hyperplasia are found during the proliferative phase. This array of morphologic alterations is known as diffuse

**Influenza A Strain-Dependent Pathogenesis in Fatal H1N1 and H5N1 Subtype Infections of Mice**

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alveolar damage. Moreover, mice infected with the 1918 influenza virus or with a recent subtype H5N1 human isolate also show considerable similarities in overall lung cellularity, composition of lung immune cell subpopulation, and cellular immune temporal dynamics (4). On the basis of these mostly retrospective studies, the pathogenesis of influenza-associated ARDS is widely viewed as being the same whatever the infecting strain.

In this study, we closely monitored ARDS in mice, caused by inoculation of identical doses of 2 different influenza strains rendered highly pathogenic toward mice by adaptation. The 2 strains elicited dramatically different disease courses and histopathologic signatures, although both strains caused death in 100% of those infected, evoked the expected diffuse alveolar damage, and led to comparable virus titers in the lungs. The pathogenesis underlying influenza-associated fatal ARDS thus depended on the infecting strain.

Materials and Methods

Animals

Eight-week-old female FVB/J mice weighing 20–25 g were obtained from Charles River Laboratories (L’Arbresle, France). Challenge studies were conducted under Biosafety Level 3 laboratory conditions and in facilities accredited by the Belgian Council for Laboratory Animal Science, under the guidance of the Institutional Animal Care and Use Committees of the Veterinary Agrochemical Research Center and University of Liège. The mice were housed in microisolator cages ventilated under negative pressure with HEPA-filtered air. The light/dark cycle was 12/12 h, and the animals were allowed free access to food and water. Before each inoculation or euthanasia procedure, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (30 mg/kg).

Viruses

Two influenza A virus strain subtypes that had low pathogenicity for laboratory mice were used in this study: a clade 1 avian influenza virus (H5N1) (A/crested_eagle/Belgium/1/2004), and a porcine influenza virus (H1N1) (A/swine/Iowa/4/76). Both viruses were first propagated in the allantoic cavity of 10-day-old embryonating hen eggs and then adapted to the mice by lung-to-lung passaging. At each passage, a set of mice were inoculated intranasally with 50 μL of either allantoic fluid or lung homogenate containing influenza A virus. At 5 days postinoculation (dpi), the mice were killed humanely by an overdose of pentobarbital, followed by exsanguination. The lungs were combined and homogenized in phosphate-buffered saline (PBS)–penicillin-streptomycin, the homogenates were centrifuged at 3,000 g for 10 min, and the supernatant was used for the next passage. The process was stopped when the mice showed a substantial loss of bodyweight on 4 dpi. This occurred after 5 (H5N1) or 31 (H1N1) passages. Lung homogenates from the last passage were homogenized and divided into aliquots for direct use in pathotyping studies, and their titers were determined by standard plaque (subtype H1N1) or median tissue culture infective dose assays (H5N1). Serial dilutions of each adapted virus stock were then injected into FVB/J mice, and the 50% mouse lethal dose (MLD₀) was calculated according to the method of Reed and Muench (5).

Pathotyping Studies

For assessment of virus-induced pathogenicity, 2 series of mice were inoculated intranasally with 10 MLD₀ of virus by instillation of 50 μL of diluted stock. Mice were monitored daily for changes in bodyweight to assess virus-induced illness. At selected intervals, 5 (virus titration or histopathology) or 10 (virus titration + dry/wet weight ratio) mice were given an overdose of sodium pentobarbital and exsanguinated by cutting the brachial artery. Lungs and pieces of heart, liver, spleen, pancreas, kidney, brain, and adipose tissue from 5 mice were fixed in 4% neutral-buffered, ice-cold paraformaldehyde, routinely processed, and embedded in paraffin for histopathologic evaluation. Five-micrometer sections were stained with hematoxylin and eosin (HE) or periodic acid–Schiff (PAS) for lesion detection. For virus detection, sections were stained with a streptavidin-biotin complex immunoperoxidase method. An in-house immunoglobulin (Ig) G–purified polyclonal rabbit antiserum raised against recombinant influenza virus nucleoprotein was used as the source of primary antibodies, and horseradish peroxidase (HRP)–conjugated anti–rabbit IgGs (Dako, Glostrup, Denmark) were used as secondary antibodies. Peroxidase was indicated by the bright red precipitate produced in the presence of 3-aminobenzidine and 9-ethyl-carbazole, and sections were counterstained with Mayer hematoxylin. For virus titrations, lungs from 5 mice were weighed, homogenized in 1 mL PBS, and clarified. The supernatants were used for virus titration by plaque or median tissue culture infectious dose assays. Because the appearance of a biphasic expiratory pattern has been shown to announce death within ≈24 h (6), this qualitative sign was chosen, for humane reasons, as the endpoint of the experimental disease. On this endpoint day, lungs from 5 mice were sampled and weighed, and homogenates thereof were desiccated for dry weight determination.

Results

Clinical, Gross Pathologic, and Virologic Observations

The influenza A virus strains (subtypes H1N1 and H5N1) used in this study were isolated, respectively, from
Influenza Strain-Dependent Pathogenesis

Influenza A virus subtype H5N1 (A) and H1N1 (B) strains on bodyweight gain or loss after intranasal inoculation of 10× the 50% mouse lethal dose on day 0. Relative values are given, as calculated with respect to preinoculation control values (mean ± SD). For each virus strain, means significantly different from baseline are indicated (Student t test for paired values). *p<0.05; †p<0.01.

Figure 1
capillaries, margined intracapillary neutrophils, necrosis of the alveolar epithelium, interstitial and alveolar edema, hyaline membranes, and invasion of the alveoli by (mostly) mononucleate cells. On the other hand, we did not observe cuboidalization of the alveoli (hyperplasia of type II pneumocytes) or hyperplasia or squamous metaplasia of the airway epithelia. These results indicate extremely rapid disease progression, nearly complete elimination of type II pneumocytes, or both. Despite these similarities, when sections of lung tissue samples taken on the last day from infected mice were pooled by subtype, an examiner unaware of which infection he was looking could easily distinguish one from the other (Figures 4, 5). The criteria for attributing lung lesions to the subtype H1N1 strain were the following: 1) earlier and much more extensive degeneration, necrosis, and desquamation of the airway epithelium; 2) a much higher cell density of the peribronchial, peribronchiolar, interstitial, and intra-alveolar infiltrates; 3) the presence of dense cuffs of mononucleate cells around the arterioles; 4) far less extensive alveolar edemas; and 5) the rarity of alveolar hemorrhages. The lesions caused by the subtype H5N1 strain were distinguishable by the late and mild regressive alterations of the airway epithelium, the extent of alveolar edema, the low cell density of the inflammatory infiltrates, the high number of alveolar hemorrhage foci, and the unusual appearance of the pulmonary arterioles (which seemed to have been dissected from the surrounding tissues because of the magnitude of the perivascular edema).

On the other hand, no arteriole showed any cuff of infiltrated mononucleate cells. Some blood-vessel walls also showed hemorrhage inside the muscle layer. No other organ examined was found to carry any histopathologic lesions except, notably, the liver in subtype H5N1–infected mice (Figure 5). These livers displayed multifocal necrosis, with necrotic foci consisting of aggregates of hypereosinophilic, pyknotic, and caryorhectic hepatocytes, admixed with a few neutrophils and lymphocytes. Such foci were
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Numerous PAS-positive islets were detected throughout the livers of subtype H5N1–infected animals, each overlapping with a necrotic focus. Patterns of centrolobular, hydropic, granular (2 dpi), centrolobular (3 dpi), and panlobular (4 dpi) microvesicular fatty degeneration were also observed in the livers of all subtype H5N1-infected animals. Interstitial hemorrhages were seen in the renal medulla.

Detection of Viruses in Tissues

The results of immunohistochemical tests were homogeneous for mice infected with the same strain. Overall, they showed that the subtype H1N1 strain swarmed centrifugally from the bronchioles throughout the lungs over 4–5 days, but remained strictly confined to the lungs. The subtype H5N1 virus, in contrast, conquered the whole lung over 24–48 hours; infected some bronchioles only later; and spread to the liver, pancreas, kidneys, spleen, brain, and perivisceral fat.

Topologic Distribution of Subtype H1N1 Antigens over Time

The virus was first detectable in the epithelium of the bronchi and bronchioles on 3 dpi. By 5 dpi, the stain was more conspicuous and appeared also in the alveolar epithelium of the areas adjacent to the airways. By 7 dpi, the virus was detectable in the epithelia of almost all bronchi and bronchioles and in the alveolar epithelium in extensive areas of the lungs. In the alveolar structures, staining showed the virus in type I and type II pneumocytes and in alveolar macrophages (Figure 6, panels A, C, and E). Nonrespiratory organs sampled on 3, 5, or 7 dpi remained strictly virus negative.

Topologic Distribution of Subtype H5N1 Antigens over Time

The virus was detectable from 2 dpi in some type II pneumocytes in peribronchiolar alveoli, some interstitial/alveolar macrophages, and some endothelial cells in the vicinity of the positive alveoli. In contrast, no nonrespiratory organ examined showed any virus-positive cells. By 3 dpi, staining of the airway epithelium was still discrete and limited, whereas the alveolar epithelium showed more pronounced staining, diffusely distributed throughout the lung. In the liver, multiple nests of positive hepatocytes were detectable, corresponding exactly with the above-mentioned necrotic PAS-positive foci. A few renal tubular epithelial cells were also positive. On 4 dpi, the alveolar epithelium was still diffusely stained, but more intensely than on 3 dpi. For the first time, staining of the bronchiolar epithelium was also visible, but not all bronchioles—far from all, in fact—showed this staining. Type II pneumocytes and alveolar macrophages were more often positive than type I pneumocytes (Figure 6, panels B, D, and F). The appearance of the kidneys and liver was the same as on 3 dpi, with more conspicuous staining. Additionally, virus-

Figure 4. Photomicrographs of the lung sections of influenza A virus (H1N1)– and (H5N1)–infected mice at endpoint (hematoxylin and eosin stain). Dramatically different histopathologic signatures are observed, with either a mostly cellular reaction (H1N1) or a mostly humoral reaction (H5N1). Panels A, D, and G: 3 views of vehicle-infected lungs (original magnification ×100). Panels B and E, subtype H1N1: Dense granulocytic and lymphocytic cell infiltrates in the interstitium and around vessels and airways with focally denuded lamina propria due to epithelial necrosis and desquamation (original magnification ×100). Panel C, subtype H5N1: Airway epithelium is intact; note the striking difference in the number of infiltrated inflammatory cells between subtypes H1N1- and H5N1-infected lungs. Dramatic congestion of the vessels is visible, with extensive interstitial and alveolar edema (original magnification ×100). Panel F, subtype H5N1: Alveoli are completely filled with edema and hemorrhages; cellular infiltrates are conspicuously absent (original magnification ×200). Panel H, subtype H1N1: An airway with a totally denuded lamina propria is shown (top, left), with its lumen filled with granulocytic and lymphocytic exsudate (original magnification ×200). A prominent peribronchial lymphocytic cuff is visible (bottom right). Panel I, subtype H5N1: Moderate inflammatory cell infiltrate, with no cuffing of any airway or vessel; an airway with a still intact epithelium is shown, located just beside a vessel with dramatic peripheral edema (original magnification ×200).
positive glial cells, splenic macrophages, cardiomyocytes, islets of Langerhans cells, and peritoneal adipocytes were also detected (Figure 7).

**Discussion**

Two influenza A viruses of different subtypes, derived from different species and showing no pathogenicity toward mice, were forced to evolve by serial passaging in mouse lungs. The 2 adapted viruses obtained showed practically identical virulence levels, with similar MLD₅₀ values. On the basis of this criterion, they appear to be more virulent than most other viruses used to date in murine models (4,8–16). Their virulence is of the same order of magnitude as those of the A/Vietnam/1203/2004 (H5N1) and A/Vietnam/1204/2004 (H5N1) viruses, whose respective MLD₅₀s are 0.7 and 2.1 PFUs (17). In both cases, inoculation of 10 MLD₅₀ causes biphasic weight loss, culminating in death with a loss of ≈10% (H5N1) or ≈25% (H1N1) bodyweight. Viral amplification is maximal for both viruses on 4 dpi, roughly corresponding to the typical inoculation-to-peak lag of natural murine respiratory viruses (6,18). On the other hand, the 2 viruses adapted in the lungs showed replication kinetics that differed substantially from what is observed with natural viruses, with a quasi-plateau from 2 to 5/6 dpi instead of the classical Gaussian profile. Notably, this peculiar amplification kinetics profile has been described previously for mice infected with mouse-adapted forms of the A/Puerto Rico/8/34 (H1N1) virus (19), the A/South Carolina/1/18 (H1N1) virus (4), and several human subtype H5N1 strains showing high or low pathogenicity (4,14). These reports suggest that this profile is typical of influenza virus amplification by the murine respiratory system.

A final common feature of infection with the 2 virus subtypes was diffuse alveolar damage, which dominates both histopathologic profiles; these results corroborate the pathologic data found in the literature. Seasonal human influenza epidemics typically consist of a transient tracheobronchitis caused by preferential attachment of the virus to the laryngeal, tracheal, and bronchial epithelia. In contrast, those influenza viruses which are highly pathogenic toward humans, from the pandemic viruses of 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) to the subtype H5N1 strains isolated from humans since 2003, additionally colonize the bronchiolar and alveolar epithelia, preferentially or not, and cause diffuse alveolar damage as an additional primary lesion (20–23). The same lesion has been found in experimental animals injected with a recent subtype H5N1 strain (14,24–26).

Although both viruses share the same pathogenicity, replication kinetics, and concentration peak, and although they both evoke diffuse alveolar damage by the endpoint day, they differ dramatically in terms of the ARDS course and pathologic signature. Flagrant differences make it easy to distinguish infections by the 2 subtypes. In subtype H1N1 infection, the disease becomes fatal at a point when the pulmonary edema is much less intense and leaves a histopathologic picture characterized by much more dense inflammatory cell infiltrates, generating cuffs around the bronchioles and blood vessels. Second, subtype H1N1 colonizes the epithelia of both the upper and lower airways, without any obvious preference, whereas subtype H5N1
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remains confined essentially to the alveoli and terminal bronchioles. Within the alveoli, unlike the subtype H1N1 strain, the subtype H5N1 strain shows a preferential tropism for type II pneumocytes and alveolar macrophages. Lastly, whereas subtype H1N1 remains strictly confined to the respiratory system, subtype H5N1 spreads to other organs. These differences demonstrate unambiguously that the 2 highly virulent influenza A viruses studied here cause 2 different forms of ARDS. This finding suggests that the physiopathologic data obtained when studying 1 virulent strain should not be extrapolated automatically to other strains. The observed differences also suggest that diverse constellations of critical mutations in the viral genome might lead to the same fatal result.

This work addresses the question of possible differences between 2 fatal diseases caused by influenza A viruses, although some previous evidence that pointed in the same direction has already been reported. For example, the pandemic human strains of 1918, 1957, and 1968, on the one hand, and the recent subtype H5N1 strains, on the other, show different tropisms: panepithelial for the former strains (20,27,28) and limited to the bronchiolar and alveolar epithelia for the latter strains, a result compatible with our own observations on mouse-adapted viruses. Likewise, a panepithelial tropism has been observed for the A/South Carolina/1/18 (H1N1) virus in mice (29), whereas a preference for the bronchioles and alveoli has been noted for recent subtype H5N1 strains that have been injected into macaques, mice, ferrets, and cats (14,25,30–37). In addition, the observed strict confinement of our subtype H1N1 strain to the respiratory system confirms previously reported data that refute the existence of polysystemic dissemination of non-H5 viruses that are lethal to humans or laboratory animals (20,27,29,38). Conversely, our observation that the subtype H5N1 strain spreads beyond the respiratory system confirms similar observations of both humans (22,22,39) and laboratory animals (14,24,25,30–34).

Although other subtype H5N1 and subtype H1N1 viruses infect other susceptible hosts, they may not show trends similar to those observed here. These results, when integrated with the diverse pieces of evidence reported elsewhere, suggest that fatal infections caused by different highly virulent influenza A viruses do not necessarily share the same pathogenesis. To be convinced, one has only to
note the ease of distinguishing, in the absence of any virus labeling, the histopathologic sections typical of the 2 strains used here (Figure 4). These different histopathologic signatures and different pathogeneses probably reflect the presence of specific sets of virulence markers that will have to be decrypted to anticipate the emergence of a pandemic. In this respect, sequence analysis of both strains will lead to insight on specific residues that are relevant for the adaptation and virulence of an influenza strain in a new host.

Furthermore, the differences between these 2 strains suggest that >1 universal cytokine storm underlies fatal influenza diseases. Thus, it might be advantageous to tailor the therapeutic approach to the influenza virus pathotype.

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Dr Garigliany is a research fellow of the Fonds National de la Recherche Scientifique, Brussels, Belgium. His research interests include influenza virus biology and host–virus interactions.

References

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## Appendix Table. Histological alterations recorded after inoculation of mice with 10 50% mouse lethal doses of 2 influenza virus strains

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Subtype H1N1 strain</th>
<th>Subtype H5N1 strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3 pi</td>
<td>Day 5 pi</td>
</tr>
<tr>
<td>Bronchi and bronchioli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>Mostly granulocytic exsudate, no blood</td>
<td>Granulocytic/lymphocytic exsudate, no blood</td>
</tr>
<tr>
<td>Epithelium</td>
<td>Partial necrosis + desquamation, all airways involved</td>
<td>Complete necrosis + desquamation, all airways involved</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>Infiltration, granulocytic, lymphocytic, macrophagic, all airways involved</td>
<td>Like day 3 pi, but infiltrations more severe</td>
</tr>
<tr>
<td>Submucosa (nonalveolar interstitium)</td>
<td>Moderate infiltrations, lymphocytic, all airways involved, no cuffs</td>
<td>Like day 3 pi, but infiltrations more severe</td>
</tr>
<tr>
<td>Arteries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wall</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Perivascular area</td>
<td>Moderate lymphocytic infiltration, many arteries involved</td>
<td>Marked lymphocytic infiltration, many arteries involved</td>
</tr>
<tr>
<td>Interstitium</td>
<td>Slight lymphocytic and macrophagic infiltration, only airway-adjacent acini involved</td>
<td>Like day 3 pi but more congestion and airway-centered foci tend to spread and coalesce</td>
</tr>
<tr>
<td>Alveolar ducts, sacs, and acini</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>No edema/hemorrhage, slight lymphocytic and macrophagic infiltration, only airway-adjacent acini involved</td>
<td>Like day 3 pi but mostly macrophage accumulation, airway-centered foci tend to spread and coalesce</td>
</tr>
<tr>
<td>Epithelium</td>
<td>No necrosis seen</td>
<td>No necrosis seen</td>
</tr>
<tr>
<td>Interstitium</td>
<td>Slight lymphocytic and macrophagic infiltration, only airway-adjacent acini involved</td>
<td>Like day 3 pi but more congestion and airway-centered foci tend to spread and coalesce</td>
</tr>
<tr>
<td>Nonrespiratory tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>No lesion seen</td>
<td>No lesion seen</td>
</tr>
<tr>
<td>Liver</td>
<td>No lesion seen</td>
<td>No lesion seen</td>
</tr>
<tr>
<td>Spleen</td>
<td>No lesion seen</td>
<td>No lesion seen</td>
</tr>
<tr>
<td>Organ</td>
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<td>No lesion seen</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Kidney</td>
<td>No lesion seen</td>
<td>No lesion seen</td>
</tr>
<tr>
<td>Brain</td>
<td>No lesion seen</td>
<td>No lesion seen</td>
</tr>
</tbody>
</table>