MAJOR ARTICLE

The Molecular Basis of the Pathogenicity of the Dutch Highly Pathogenic Human Influenza A H7N7 Viruses

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During the highly pathogenic avian influenza (HPAI) H7N7 virus outbreak in The Netherlands in 2003, 88 infected persons suffered from mild illnesses, and 1 died of pneumonia. Here, we studied which of the 14 amino acid substitutions observed between the fatal case (FC) virus and a conjunctivitis case (CC) virus determined the differences in virus pathogenicity. In virus-attachment experiments, the CC and FC viruses revealed marked differences in binding to the lower respiratory tract of humans. In a mouse model, the hemagglutinin (HA) gene of the FC virus was a determinant of virus tissue distribution. The lysine at position 627 of basic polymerase 2 (PB2) of the FC virus was the major determinant of pathogenicity and tissue distribution. Thus, remarkable similarities were revealed between recent HPAI H5N1 and H7N7 viruses. We conclude that the influenza virus HA and PB2 genes should be the prime targets for molecular surveillance during outbreaks of zoonotic HPAI viruses.

The last decade has seen a marked increase in the number of outbreaks caused by highly pathogenic avian influenza (HPAI) viruses in domestic birds, with subsequent transmission of some of these viruses to wild birds and several mammalian species, including humans [1–6]. The low-pathogenic avian influenza (LPAI) H5 and H7 viruses circulating in wild birds are the progenitors of HPAI viruses [7, 8]. The switch from the LPAI phenotype to the HPAI phenotype in poultry is predominantly determined by the introduction of basic amino acids in the cleavage site of hemagglutinin (HA). The molecular basis of the pathogenicity of HPAI viruses in humans is still poorly understood. Recent studies have suggested that the ability of virus to bind to sialic acid (SA) receptors present on cells of the new host [9, 10], efficient replication in these cells [11–13], and evasion of the host immune response [14, 15] contribute to pathogenicity.

During an HPAI H7N7 outbreak in The Netherlands in 2003, viruses were transmitted to humans in close contact with infected poultry. Of 89 patients with laboratory-confirmed cases of human H7N7 infection, most suffered from conjunctivitis, and a few had mild influenza-like illness. One veterinarian died as the result of pneumonia, acute respiratory distress syndrome, and related complications [16, 17]. Infection of the human eye resulting in conjunctivitis has been described previously for H7 influenza A viruses [18]. This may be attributed to the α2,3-linked SA binding preference of avian influenza A viruses and the presence of α2,3-linked SA on epithelial cells in the human cornea and conjunctiva, in contrast to the predominant presence of α2,6-linked SA in the human upper respiratory tract [19].

Sequence analyses of the virus isolated from the patient with the fatal H7N7 case (influenza A/Nether-

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lands/219/03) revealed 14 amino acid substitutions relative to viruses isolated from chickens and from the patients with conjunctivitis (such as influenza A/Netherlands/33/03 CC) in 5 different gene segments, basic polymerase 2 (PB2; 5 substitutions), acidic polymerase (PA; 1), HA (3), neuraminidase (NA; 4), and nonstructural (NS; 1) [16]. One or more of these substitutions might have been responsible for the higher virus pathogenicity and the fatal outcome of the infection.

Here, we describe the patterns of attachment of 2 prototype HPAI H7N7 viruses from the Dutch HPAI outbreak to human ocular and respiratory tissues and study the molecular determinants of the pathogenicity of these viruses in a mouse model. The increased understanding of the molecular determinants of efficient replication and spread of avian viruses in humans may lead to a targeted monitoring of HPAI viruses during future zoonotic influenza outbreaks.

METHODS

Viruses. Influenza A/Netherlands/33/03(H7N7) and influenza A/Netherlands/219/03(H7N7) were isolated from a patient with conjunctivitis and from the patient with the fatal case, respectively, during the Dutch H7N7 outbreak [16]. On the basis of sequence analyses, the selected conjunctivitis case (CC) virus was a good representative of virus isolates obtained from the patients with conjunctivitis [16]. The gene segments of the CC and fatal case (FC) viruses were amplified by reverse-transcription polymerase chain reaction, and cloned and recombinant viruses were generated by reverse genetics as described elsewhere [20] and propagated in embryonated chicken eggs. We generated recombinant FC and CC viruses and reassortant viruses consisting of 7 gene segments of the CC virus and 1 of the FC virus (CC-FC PB2, CC-FC PA, CC-FC HA, CC-FC NA, and CC-FC NS). Mutant viruses containing an E627K mutation in PB2 of the CC virus (CC-PB2 E627K) or a K627E substitution in PB2 of the FC virus (FC-PB2 K627E) were also produced. The genotypes of the recombinant viruses were confirmed by sequencing.

Virus histochemistry. Archival paraffin-embedded human and animal tissue sections were obtained from the Erasmus Medical Center Departments of Pathology and Virology, respectively. Ocular tissues were obtained from the Cornea Bank Netherlands Ophthalmic Research Institute. Three donors were used for each tissue. All tissues were histologically normal by microscopic examination of hematoxylin-eosin (HE)–stained sections and were obtained from humans or animals with no evidence of respiratory tract infection. Virus histochemistry using the FC and CC viruses was performed as described elsewhere [10, 21]. After concentration and purification of virus stocks by use of sucrose gradients, viruses were inactivated by dialyses against 0.1% formalin and labeled with an equal volume of 0.1 mg/mL fluorescein isothiocyanate (FITC; Sigma-Aldrich).

Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with alcohol. FITC-labeled in-

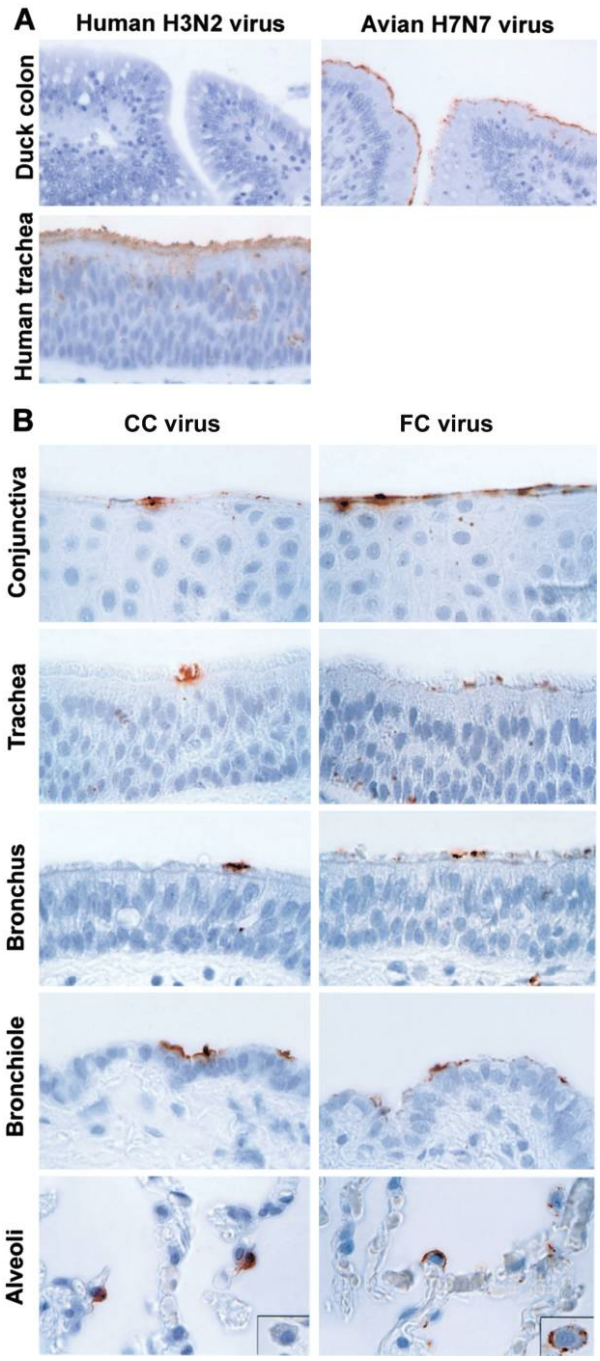

Figure 1. In vitro attachment of the conjunctivitis case (CC) virus and the fatal case (FC) virus to human ocular and respiratory tract tissues. Control staining of prototype human and avian influenza A viruses to duck colon and human trachea is shown in panel A. Virus attachment is visible as red staining. In panel B, both viruses attached to epithelium of human conjunctiva, trachea, bronchi, and bronchioles. Sections were counterstained with hematoxylin. Original magnification, ×100.

fluenza viruses (50–100 hemagglutination units) were incubated with the tissues overnight at 4°C. The label was detected with a peroxidase-labeled rabbit anti-FITC (Dako). Tissues were counterstained with hematoxylin and embedded in glycerol gelatin (Merck).

To validate the method, we incubated labeled FC virus and H3N2 virus (influenza A/Netherlands/213/03) with human trachea and mallard intestine. The FC virus bound abundantly to epithelial cells in duck intestine and poorly to human trachea, whereas this was reversed for the H3N2 virus (figure 1).

Positively staining type 1 and 2 pneumocytes and macrophages in alveoli were counted in 10 arbitrarily chosen high-power fields (×40) for all 3 tissue donors. Positively staining nonciliated cuboidal cells in the bronchiole were also counted in ×40 fields for all 3 tissue donors, but the number of available fields was limited to 7 for 1 donor and to 1 for the other 2 donors.

Mouse model. Groups of six 6–8-week-old female BALB/c mice (Harlan) were inoculated intranasally with H7N7 virus. The mice were observed for clinical signs and weighed twice daily as an indicator of disease. Three days after inoculation, 3 mice from each group were killed, and virus titers in the lungs, spleen, liver, kidneys, and brain were determined. The other 3 mice were killed on day 7 or were euthanized after the development of severe disease or discomfort, in agreement with national animal welfare regulations. Intranasal inoculations and euthanasia were performed under anesthesia with inhaled isoflurane. Animal studies were approved by an independent animal ethics committee and were performed under biosafety level 3+ conditions.

Virus titrations. Viruses were titrated by end-point dilution in MDCK cells [20]. Lungs, spleen, liver, kidneys, and brain were collected and homogenized [22], and 10-fold serial dilutions of tissue homogenates were used to inoculate MDCK cells. Three days after inoculation, the infected cell supernatants were tested for agglutinating activity using turkey erythrocytes. Infectious titers were calculated from 5 replicates by the Spearman-Karber method [23].

Histopathological analysis and immunohistochemistry. Histopathological analysis and immunohistochemistry were performed in mice inoculated with the FC, CC, CC-FC PB2, CC-FC HA, CC-PB2 E627K, and CC-PB2 K627E viruses. Four mice were killed 3 days after inoculation by exsanguination under general anesthesia with isoflurane. Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained with HE for histological evaluation or with a monoclonal antibody against the nucleoprotein (NP) of influenza A virus for immunohistochemistry [24]. Trachea, lungs (after inflation with 10% neutral-buffered formalin in situ; cross-sections of the left lobe and the right cranial, medial, and caudal lobes were obtained), brain, liver, kidneys, and spleen were examined. For semiquantitative assessment of influenza virus–associated inflammation in the respiratory tract, HE-stained sections of trachea and lungs were examined for the presence of epithelial necrosis and infiltration by inflammatory cells and then scored as mild (0%–10% of tracheal mucosa, bronchiolar cross-sections, or alveolar area affected), moderate (10%–50% affected), or severe (50%–100% affected).

RESULTS

H7N7 virus attachment. We investigated the role played by human conjunctiva as potential *porte d'entrée* for H7N7 influenza A viruses. Using virus histochemistry [10, 21], we studied the attachment of the FC and CC viruses to human ocular tissues, including cornea and the bulbar conjunctiva. Both viruses attached to the epithelium of the cornea and conjunctiva (figure 1). The human ocular tissues may thus represent a site of entry for H7N7 viruses. This was shown for both the CC and the FC virus, despite the fact that the FC virus was isolated from the lower respiratory tract (LRT) and that no virus was detected in the patient's conjunctiva swabs [16].

To determine whether differences in patterns of virus attachment could explain the difference in disease outcome for the CC and FC viruses, we compared their patterns of attachment to human trachea and tissues of the LRT (bronchus, bronchiole, and alveoli). There was no difference in virus attachment to trachea or bronchus (figure 1). Attachment to few ciliated epithelial cells was observed, with more-abundant binding to epithelial cells and excreted mucus of submucosal glands. In bronchioles, both viruses attached to ciliated cells and nonciliated cuboidal cells. The FC virus attached more abundantly to nonciliated cuboidal cells in sections from 1 of 3 tissue donors; although the CC virus on average attached to 8 cells/×40 field, the FC virus attached to 40 cells/×40 field. In alveoli, the CC and FC viruses on average attached to 1.13 (range for the 3 donors, 0.9–1.3) and 1.23 (range, 0.9–1.8) type 1 pneumocytes/×40 field, respectively. The FC virus attached more abundantly to type 2 pneumocytes in alveoli than did the CC virus, with attachment to 5.7 (range, 4.0–6.6) and 2.6 (range, 2.4–2.8) cells/×40 field, respectively. The FC virus showed more-abundant attachment to alveolar macrophages than did the CC virus, with values of 1.3 (range, 0.6–2.1) and 0.1 (range, 0–0.2) positive alveolar macrophages/×40 field, respectively (figure 1). Thus, the attachment pattern of the FC virus differed from that of the CC virus in alveoli, which was the site of the primary lesion—diffuse alveolar damage—in the person who died from this infection [16]. Furthermore, the attachment patterns of the FC virus showed marked similarities to those of a recent HPAI H5N1 virus [10].

A mouse model of infection with the CC and FC viruses. We developed a mouse model to distinguish the pathogenicity of the CC and FC viruses. Groups of 6 female BALB/c mice were inoculated with the CC and FC viruses, and the mice were weighed daily and observed for clinical signs of disease. When inoculated with high virus doses, mice became severely ill and required euthanasia because of the severity of symptoms, irrespective of whether they were infected with the CC or FC virus (data not shown). Mice inoculated with 5×10^2 $TCID_{50}$ of the CC virus showed no signs of disease and lost no body weight during a 7-day observation period. Mice inoculated with the same dose of the FC virus showed signs of disease, such as loss of body weight (figure 2*A*), ruffled fur, lethargy, and respiratory problems from day 2 after inoculation onward. These mice had to be euthanized because of the severity of symptoms on day 5, whereas the mice inoculated with the CC virus survived the infection (figure 2*B*). We used a dose of 5×10^2 $TCID_{50}$ to inoculate mice in all further experiments.

On day 3, 3 mice from each group were killed, and virus titers in the lungs, spleen, liver, kidneys, and brain were determined. In the lungs of mice inoculated with the FC virus, virus titers were >1000-fold higher than those in the lungs of mice inoculated with the CC virus (figure 3). In all mice inoculated with the FC virus, virus could be detected in spleen,

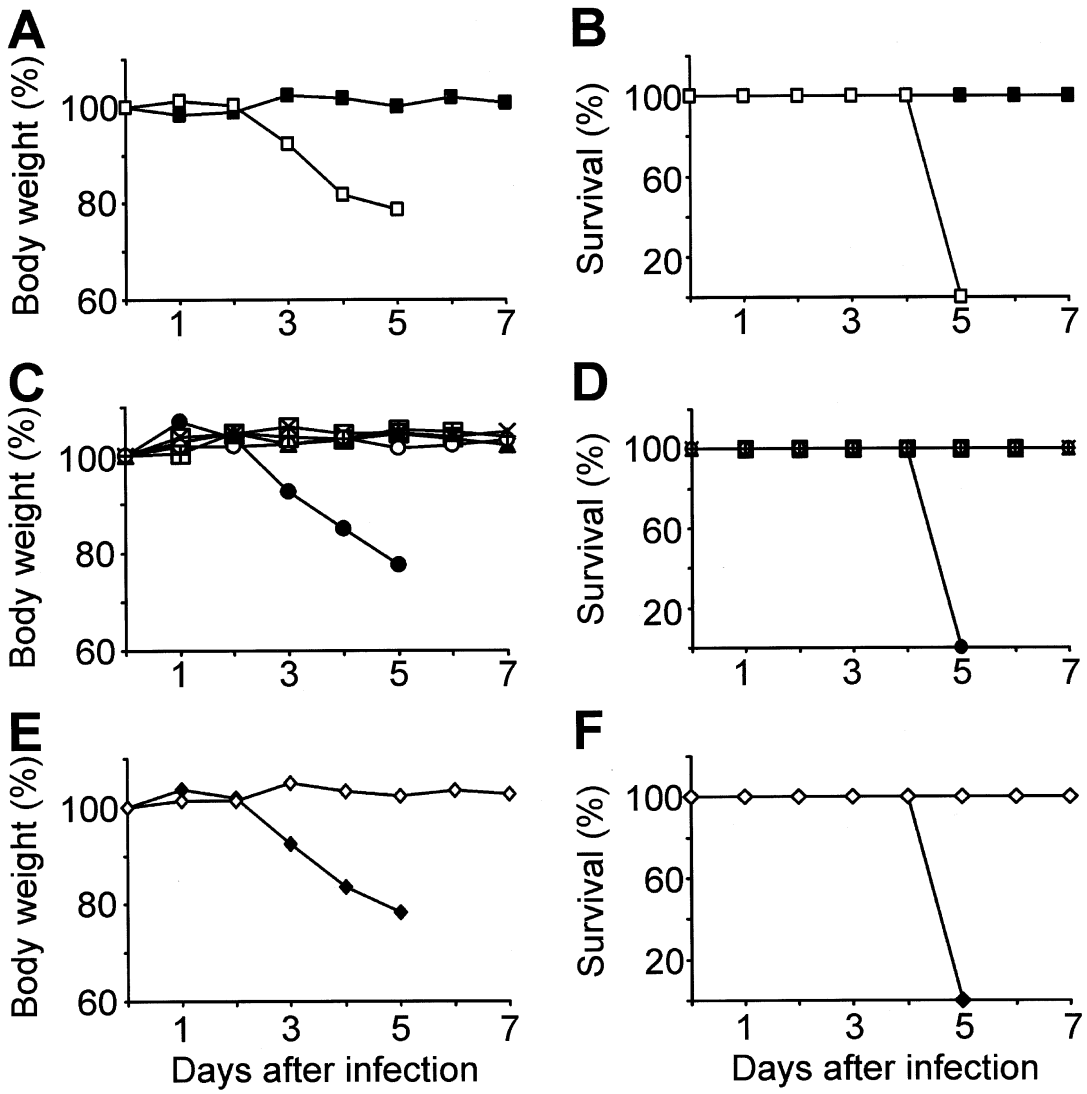

Figure 2. Loss of body weight and survival after intranasal inoculation of mice with highly pathogenic avian influenza H7N7 viruses. Groups of 6 mice were inoculated intranasally with 5×10^2 $TCID_{50}$ of the conjunctivitis case (CC; ■), fatal case (FC; □), CC-FC PB2 (●), CC-FC PA (×), CC-FC HA (▲), CC-FC NA (○), CC-FC NS (+), CC-PB2 E627K (◆), or FC-PB2 K627E (◇) virus. Mice were weighed daily, and the percentage of body weight was calculated relative to the weight at time of inoculation (*A, C,* and *E*). Mice were either euthanized because of the severity of symptoms on day 5 or were killed at the end of the experiment, on day 7 after inoculation; the percentage of mice surviving the infection is shown as a function of time (*B, D,* and *F*).

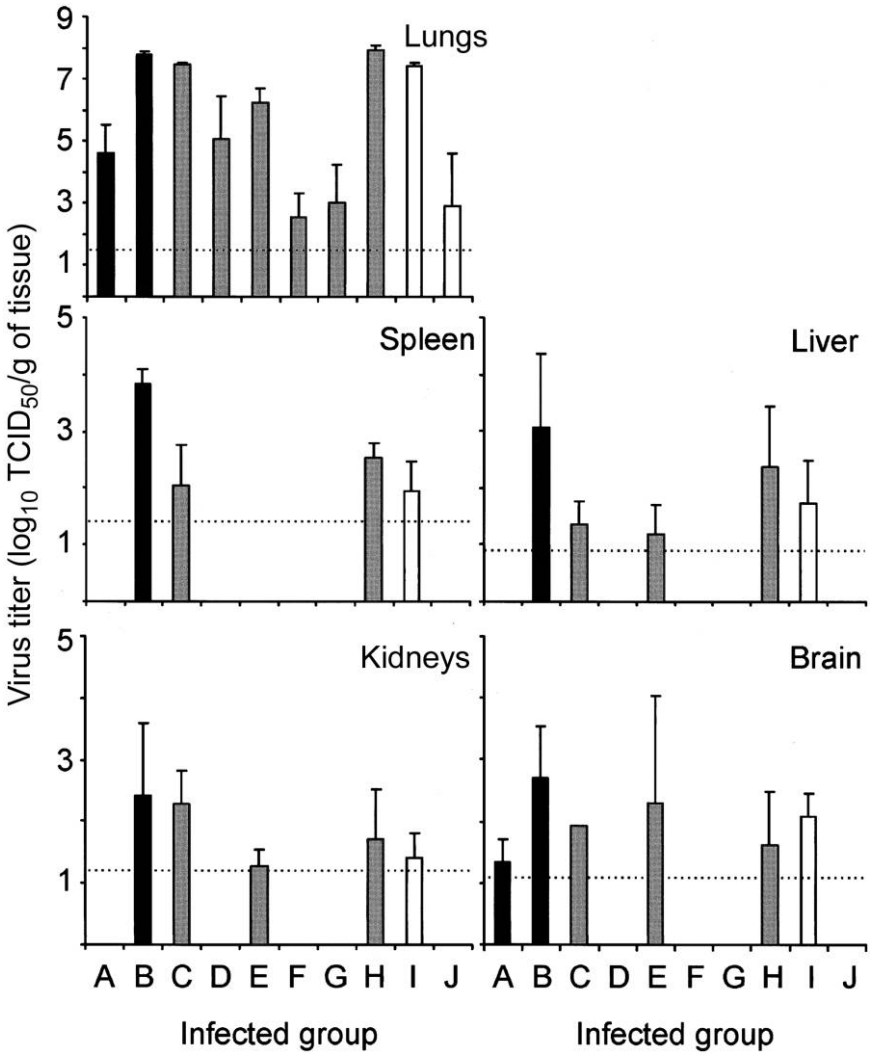

Figure 3. Virus titers in mice after inoculation with highly pathogenic avian influenza H7N7 viruses. Mice were inoculated intranasally with 5×10^2 $TCID_{50}$ of the conjunctivitis case (CC; *A*), fatal case (FC; *B*), CC-FC PB2 *(C),* CC-FC PA *(D),* CC-FC HA *(E),* CC-FC NA *(F),* CC-FC NS *(G),* CC-FC PB2 HA *(H),* CC PB2 E627K *(I),* or FC PB2 K627E *(J)* virus. On day 3, 3 mice from each group were killed, tissues were collected, and virus titers in lungs, spleen, liver, kidneys, and brain were determined in MDCK cells. The geometric mean virus titer per group of mice was calculated. To calculate the geometric mean, the cutoff value was used for negative results. Each dotted line indicates the cutoff value of the assay for each of the organs, and error bars indicate SDs. Bars are not shown when virus titers were below the cutoff value for all mice in a group. Black bars indicate wild-type viruses, gray bars indicate reassortant viruses, and white bars indicate mutant viruses.

liver, kidneys, and brain, whereas virus could not be detected outside the lungs in mice inoculated with the CC virus, except for 1 mouse in which virus was detected in the brain (figure 3, black bars, A and B). Histopathological examinations performed on 4 mice on day 3 revealed that, in the mice inoculated with the FC virus, lesions occurred throughout the respiratory tract and consisted of necrosis and inflammation (table 1 and figure 4). These lesions were most pronounced in the trachea and were progressively milder in the bronchi, bronchioles, and alveoli. Lesions in the trachea, bronchi, and bronchioles were characterized by necrosis or loss of epithelial cells, infiltration of the epithelium and of subepithelial connective tissue by neutrophils and lymphocytes, and the presence of cell debris mixed with erythrocytes and neutrophils in the lumen (figure 4). Lesions in the alveoli were centered around bronchioles and were characterized by thickening and hypercellularity of the alveolar walls and by flooding of the alveolar lumina with variable proportions of cell debris, fibrin, edema fluid, erythrocytes, neutrophils, and mononuclear cells (figure 4). No lesions were detected in brain, heart, spleen, liver, or kidneys, despite virus recovery from these organs. In the mice inoculated with the CC virus, lesions were restricted to the respiratory tract of only 1 mouse (table 1) and consisted of mild to moderate epithelial cell necrosis and infiltration by neutrophils in trachea, bronchi,

and bronchioles. No lesions were detected in any of the tissues of the sham-inoculated mice.

Expression of influenza virus antigen was limited to respiratory tract tissues, as determined by immunohistochemistry. The more-abundant presence of NP-positive cells in the lesions of mice inoculated with the FC virus corresponded to higher virus titers in the lungs of these mice (table 1 and figure 4). Influenza virus antigen expression was seen in ciliated and nonciliated epithelial cells in trachea, bronchi, and bronchioles and in type 1 and 2 pneumocytes in the alveoli. Influenza virus antigen expression was associated with the presence of histological lesions and was generally strongest at the transition of normal and necrotic tissue.

Mapping the determinants of pathogenicity of the FC virus. To determine which gene segment was responsible for the increased pathogenicity of the FC, we constructed 5 reassortant viruses consisting of 7 gene segments of the CC virus and each of the gene segments of the FC virus harboring amino acid substitutions. After inoculation, mice were weighed daily and observed for clinical signs of disease. The mice inoculated with CC-FC PB2 lost body weight and showed other symptoms of disease, whereas the mice inoculated with CC-FC PA, CC-FC HA, CC-FC NA, and CC-FC NS did not display any symptoms. The mice inoculated with CC-FC PB2 required euthanasia because of the severity of symptoms on day 5 (figure 2). As with the FC virus, the mice inoculated with CC-FC PB2 had high virus titers in the lungs (figure 3, gray bar, C). In the mice inoculated with CC-FC PA, CC-FC NA, or CC-FC NS, virus titers in the lungs were comparable to those in the mice inoculated with the CC virus. The CC-FC HA virus displayed intermediate titers in the lungs. Virus was detected in spleen, liver, kidneys, and brain of the mice inoculated with CC-FC PB2 but not of the mice inoculated with CC-FC PA, CC-FC NA, or CC-FC NS (figure 3, gray bars, C–G). CC-FC HA virus was detected in the liver, kidneys, and brain, although not all organs were positive in all mice from this group. Inoculation of mice with a double-reassortant virus, CC-FC PB2 HA, did not result in increased virus titers compared with CC-FC PB2 (figure 3, gray bars, H). By histopathological and immunohistochemical analysis, the nature and severity of the lesions caused by the CC-FC PB2 virus did not differ from those of the lesions caused by the FC virus. Lesions caused by the CC-FC HA virus were comparable to those caused by the CC virus (table 1).

Mapping the determinants of pathogenicity in PB2. Studies of HPAI H5N1 viruses have shown that, apart from the multibasic cleavage site in HA, an E627K substitution in PB2 was the main determinant of virulence [11, 12]. Because this substitution is present in the FC virus, we determined whether it is also important for the pathogenicity of HPAI H7N7 viruses. We constructed the CC virus with the E627K substitution in PB2 (CC-PB2 E627K) and the FC virus with a K627E substitution in PB2 (FC-PB2 K627E) and inoculated groups of 6 mice. This E627K substitution reversed the phenotype of the FC and CC viruses. The mice inoculated with the CC-PB2 E627K virus showed signs of disease, including loss of body weight from 2 days after inoculation onward, whereas the mice inoculated with the FC-PB2 K627E virus did not (figure 2*E*). Consequently, the mice inoculated with CC-PB2 E627K virus required euthanasia because of the severity of symptoms on day 5, whereas the mice inoculated with the FC-PB2 K627E virus survived (figure 2*F*). Lung titers on day 3 in the mice inoculated with the CC-PB2 E627K virus were comparable to those in the mice inoculated with wild-type FC virus, whereas those in the mice inoculated with the FC-PB2 K627E virus were comparable to those in the CC virus–inoculated mice (figure 3, white bars, I and J). CC-PB2 E627K virus was also detected in spleen, liver, kidneys, and brain, whereas FC-PB2 K627E was not. As determined by histopathological and immunohistochemical analysis, the FC-PB2 K627E virus caused lesions comparable to those caused by the CC virus, whereas the CC-PB2 E627K virus caused lesions comparable to those caused by the FC virus (table 1).

Table 1. Pathological analyses of respiratory tract tissues of mice infected with recombinant H7N7 viruses.

	Trachea			Bronchioles			Alveoli		
	No. of mice with		Severity	No. of mice with		Severity	No. of mice with		Severity
Virus	Antigen	Lesion	of lesion	Antigen	Lesion	of lesion	Antigen	Lesion	of lesion
Mock	0	0	NA	0	0	NA	0	0	NA
CC	2	1	Moderate	4[a]	1	Mild	1[a]	0	NA
FC	4	4	Severe	4	4	Moderate/severe	4	4	Mild/moderate
CC-FC PB2[b]	3[a]	3	Severe	3	3	Moderate/severe	3	3	Mild/moderate
CC-FC HA	2	0	NA	1	1	Mild	0	0	NA
CC-PB2 E627K	4	4	Severe	4	4	Mild/severe	4	4	Mild/moderate
FC-PB2 K627E	3[a]	1	Mild	4[a]	2	Mild	3[a]	0	NA

NOTE. CC, conjunctivitis case; FC, fatal case; NA, not applicable.

[a] Antigen could be detected in only a few cells.

[b] Only 3 mice were infected with this virus.

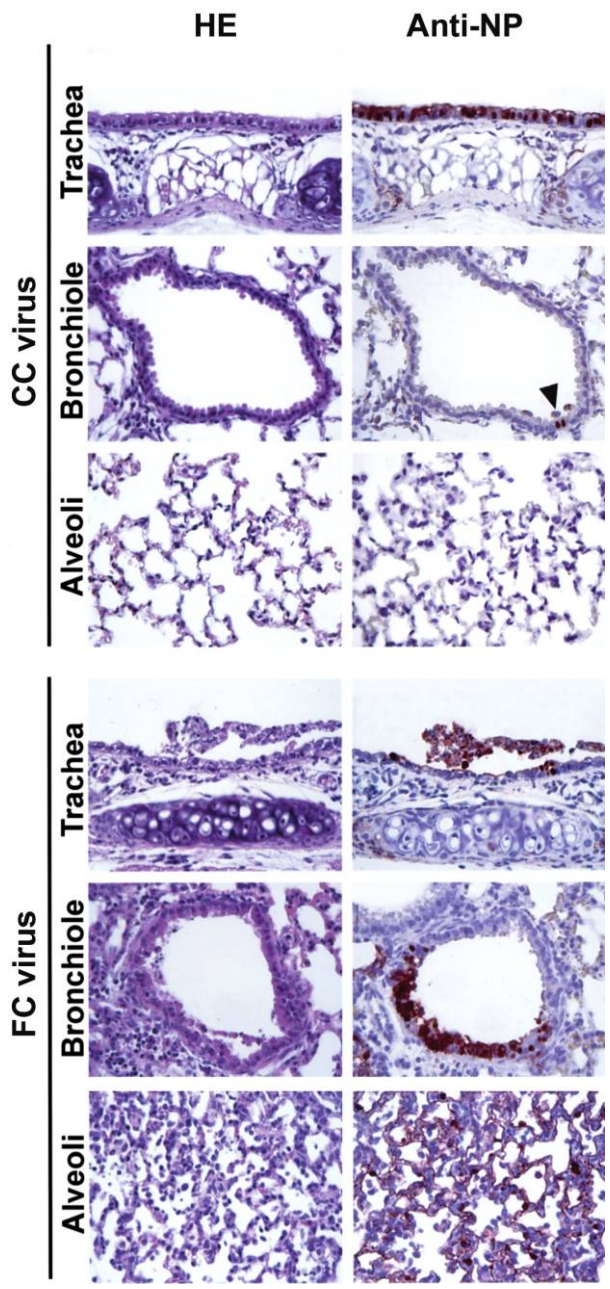

Figure 4. Histopathological and immunohistochemical analysis of respiratory tract tissues of mice infected with the conjunctivitis case (CC) virus or the fatal case (FC) virus. Histological lesions *(left)* were absent in trachea, bronchioles, and alveoli of CC virus–infected mice. Serial sections of these tissues *(right)* demonstrated abundant expression of viral antigen in tracheal epithelium, rare expression in bronchiolar epithelium *(arrowhead),* and no expression in alveoli. In contrast, marked necrotizing and inflammatory changes were seen in the same tissues of FC virus–infected mice. Serial sections of these tissues showed that these lesions were closely associated with abundant expression of influenza virus antigen. Mice were inoculated with 5×10^2 $TCID_{50}$ of the CC or the FC virus and killed 3 days later. Tissue sections were stained with hematoxylin-eosin (HE) or with a monoclonal antibody against nucleoprotein (anti-NP). Original magnification, ×100.

DISCUSSION

The closely related FC and CC viruses from the Dutch H7N7 outbreak offered a unique opportunity to study determinants of pathogenicity of H7 viruses in in vitro and in vivo model systems. In mice, we could distinguish between the pathogenicity of the CC and the FC virus. Although both viruses were lethal at high doses, the FC virus was more pathogenic at a low dose. At a dose of 5×10^2 $TCID_{50}$, the CC virus did not induce disease, whereas infection with the FC virus was lethal. Moreover, the FC virus was detected in spleen, liver, kidneys, and brain, whereas the CC virus was confined to the lungs. Histopathologically, clear differences were observed in the respiratory tract. Mild to moderate lesions were detected in only 1 of 4 mice at day 3 after inoculation with the CC virus, whereas with the FC virus severe lesions were detected in all mice. These data indicate that the FC virus is intrinsically more pathogenic than the CC virus and could explain why the patient infected with the FC virus presented with such severe symptoms and died.

By producing single-gene reassortant and mutant viruses between the CC and the FC virus, we showed that the PA, NA, or NS genes did not determine the pathogenicity of the FC virus in mice. Virus titers in the lungs of the mice inoculated with CC-FC HA were higher than those in the mice inoculated with the CC virus, and the virus was detected outside the lungs, indicating that the FC HA gene contributed to enhanced virus replication and tissue distribution. We could not determine whether the effect of HA on the spread of virus to different organs was due to altered receptor specificity/affinity or merely resulted from higher virus titers in the lungs in a receptor-independent mechanism. By virus histochemistry, the FC virus showed patterns of attachment to human respiratory tissues that could partly explain its pathogenicity. Both the CC and the FC virus showed limited binding to tracheal epithelium, in contrast to a human H3N2 virus, which bound abundantly to this tissue (figure 1) [10]. The FC virus attached more abundantly than did the CC virus to nonciliated cuboidal cells in the bronchioles and to alveolar macrophages and type 2 pneumocytes in the alveoli. These subtle differences in attachment between the CC and the FC virus in the LRT of humans could indicate differences in specificity or affinity for the SA residues present in the human host. The attachment pattern of the FC virus to the human respiratory tract showed great similarity with that observed with influenza A/Vietnam/1194/04, a recent HPAI H5N1 isolate [10], and is in agreement with the distribution of α2,3-linked SA in the human respiratory tract [9]. The attachment patterns of influenza A/Vietnam/1194/04 and the FC virus correspond to pathological findings in patients with fatal cases of H5N1 and H7N7 infection [1, 16], which show diffuse alveolar damage in the lower pulmonary lobes. It has been shown that HPAI H5N1 viruses are potent inducers

of the production of proinflammatory cytokines by macrophages [25], and it was suggested that this cytokine induction may relate to the unusual disease severity caused by HPAI H5N1 viruses in humans [25]. The abundant attachment of the FC virus to alveolar macrophages may imply that similar mechanisms increase the pathogenicity of the FC virus.

When the CC and the FC virus were compared, the main determinant of pathogenicity of the FC virus in mice was PB2—more specifically, the lysine at position 627. Inoculation of mice with a double-reassortant virus, CC-FC PB2 HA, did not result in increased pathogenicity relative to CC-FC PB2, given that virus could be detected in lungs, spleen, liver, kidneys, and brain, at titers comparable to those for CC-FC PB2. Pathogenicity studies using HPAI H5N1 viruses have also shown a prominent role for the lysine at position 627 of PB2 in mice [11, 12] but not in ferrets [12]. In light of the presence of this lysine in the 1918 Spanish influenza virus, in all subsequent human lineages of influenza A viruses, and in recent mammalian H5 HPAI isolates but of its absence in nearly all avian virus PB2 sequences, it is likely an important determinant of efficient virus replication in humans [26, 27].

Taken together, the data presented here lead us to suggest that the virus isolated from the patient with the fatal case either entered via the ocular epithelium and gained access to the LRT (e.g., via the lacrimal duct) or accessed the LRT directly. During the infection, the PB2 E627K substitution allowed efficient replication of this virus in the LRT, possibly aided by 1 or more substitutions in HA, leading to pneumonia, acute respiratory distress syndrome, and eventually death. Given the similarities between the findings of pathogenesis studies of zoonotic H5N1 and H7N7 viruses and the recorded changes in the pandemic influenza viruses that emerged in the last century, the HA and PB2 genes should be considered prime targets for genetic characterization during HPAI outbreaks.

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