Avian Influenza A(H5N1) Viruses Can Directly Infect and Replicate in Human Gut Tissues

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The human respiratory tract is a major site of avian influenza A(H5N1) infection. However, many humans infected with H5N1 present with gastrointestinal tract symptoms, suggesting that this may also be a target for the virus. In this study, we demonstrated that the human gut expresses abundant avian H5N1 receptors, is readily infected ex vivo by the H5N1 virus, and produces infectious viral particles in organ culture. An autopsy colonic sample from an H5N1-infected patient showed evidence of viral antigen expression in the gut epithelium. Our results provide the first evidence, to our knowledge, that H5N1 can directly target human gut tissues.

Although human infection with avian influenza A(H5N1) virus remains rare, the number of human cases has accumulated over the past 5 years, and more severe pneumonia and greater mortality (>60%) has been observed [1]. Epidemiological studies have shown that most human H5N1 cases are associated with close contact with infected poultry, and some patients have a history of consuming raw or undercooked poultry or poultry products [1]. In addition, 2 cases of human H5N1 infection have been reported in patients with severe diarrhea and encephalitis in the absence of pneumonia [2]. Transmission of avian influenza virus to tigers and cats after consumption of infected chickens has been documented [3, 4]. This raises the important issue of whether H5N1 can be orally transmitted to humans.

Human and avian influenza A viruses use different receptors for cell entry [5]. Human-adapted influenza A viruses preferentially bind to “humanlike” sialic acid (SA)–α2,6-galactose (Gal)–terminated saccharides (hereafter, SA-α2,6-Gal), whereas avian influenza A viruses prefer receptors with “avianlike” α2,3 linkages (hereafter, SA-α2,3-Gal). The absence of the avian SA-α2,6-Gal receptors in the human upper respiratory tract may be one of the key factors for limiting human-to-human transmission of H5N1 viruses [6, 7], although fresh nasopharyngeal tissues could be infected by H5N1 in the absence of SA-α2,3-Gal receptor expression [8], indicating the involvement of other binding sites.

In this study, we sought to address the questions of whether the human gastrointestinal (GI) tract could be infected by the avian influenza virus and whether it could support subsequent viral replication—a subject that remains at the center of debate. To this end, we investigated SA receptor expression in human gut tissues; furthermore, we demonstrated for the first time, to our knowledge, the ability of H5N1 viruses to infect and replicate in human GI tissues ex vivo. Evidence of viral infection and associated gut pathology in a recent postmortem sample from an H5N1-infected patient is also presented.

Methods. A human H5N1 viral isolate (A/AH/01/05) was propagated in 10-day-old embryonated chicken eggs and used in the present study. The virus stock was titrated to 1 × 104 median tissue culture infective doses (TCID50)/mL, in accordance with World Health Organization (WHO) guidelines [9].

Colonic samples were obtained from a patient who died of human H5N1 infection in China in 2008. This case was laboratory confirmed by real-time polymerase chain reaction (PCR) and virus isolation from respiratory samples, according to the WHO case definition of human H5N1 infection. Tissues obtained were fixed in 10% formalin and prepared for routine histological analysis. For ex vivo organ culture, colorectal samples were freshly obtained from patients undergoing colectomy for colon cancer (mean age, 48 years; age range, 24–68 years). Colonic samples were obtained from macroscopically and microscopically unaffected colonic areas. Routine histological analysis showed normal mucosal architecture. This study was ap-
proved by the local ethics committee, and informed consent was given by all patients in the study.

Six tissue arrays (AA8; Super Biochips) containing different GI and respiratory tissues were also used to study the distribution of SA receptors by 2-step immunofluorescent staining. This was performed on 5-μm-thick deparaffinized sections, using fluorescein isothiocyanate–conjugated SNA (Sambucus nigra lectin), which stains SA-α2,6-Gal (human); biotinylated MAA (Maackia amurensis lectin; MAA2), which stains SA-α2,3-Gal (avian) (Vector Laboratories); and streptavidin-conjugated Alexa Fluor (568 nm; Invitrogen) for detection, as described elsewhere [6, 7]. Sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride and observed under an epifluorescent microscope (Axiovert S100; Carl Zeiss).

Ex vivo culture of gut tissues from 4 individuals was performed using the mucosal layer from the colonic samples. After being washed extensively in serum-free Dulbecco modified Eagle medium (DMEM) containing antibiotics, mucosal samples were cut into 1–2-mm-thick sections before being put into a 48-well plate containing DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL ciprofloxacin monohydrochloride, and 0.5% bovine serum albumin fraction V. The mucosal tissues were infected with A/Al/01/05 virus at 1 × 10^3 TCID₅₀/mL for 2 h at 37°C in 5% CO₂. After being washed extensively to remove unbound virus, mucosal tissues were further incubated for up to 24 h, at which time the tissues were fixed in either 10% neutral buffered formalin for immunohistochemical analysis or 70% ethanol for viral RNA extraction. Virus in the supernatant was titrated using a TCID₅₀ assay, and virus in the infected tissues was quantified by quantitative real-time PCR, as described below. Adult duck (Chinese Ma) gut sample was used as a positive control for histological staining.

Viral antigen of the H5N1 virus in infected tissues was detected by means of monoclonal antibodies (mAbs) against the nucleoprotein (NP) of influenza A virus (Serotec) and a 2-step peroxidase staining kit (Dako), in accordance with the manufacturer’s instructions. Briefly, deparaffinized sections were incubated with the mAbs (1:100) or the isotype control mAb (mouse immunoglobulin G 2a) for 30 min at room temperature and washed in Tris-buffered saline before addition of biotinylated rabbit anti-mouse and peroxidase-conjugated streptavidin immunoglobulins (Catalyzed Signal Amplification System; Dako). Tissue sections were revealed by means of 3,3-diaminobenzidine solution (EnVision Detection Systems; Dako), mounted with depex polystyrene, and examined by light microscopy (Axiovert S100; Carl Zeiss).

Titration of virus in the samples by the TCID₅₀ assay was done as described elsewhere [10]. After washing the confluent Madin-Darby canine kidney (MDCK) cells 3 times with phosphate-buffered saline, samples containing viruses were diluted by 0.5 log₁₀₀₀ before being incubated with MDCK cells at 37°C in 5% CO₂ for 45 min. The supernatant was discarded to remove the unbound virus. After being washed with phosphate-buffered saline 3 times, the cells were incubated for another 72 h. The plates were observed for cytopathogenic effect daily. Virus in the supernatant was quantified by a standard hemagglutination assay using chicken red blood cells, as described elsewhere [9]. The end point of viral dilution leading to hemagglutination in 50% of inoculated wells was calculated using the Karber method.

Total RNA from the supernatant of cell culture or tissue samples was extracted using an RNeasy Mini kit (Qiagen), in accordance with the manufacturer’s instructions. Quantitative real-time PCR analysis was performed by means of a SmartCycler detection system (Cepheid), using a fluorescently labeled TaqMan probe (Applied Biosystems) to enable continuous monitoring of amplification. The sequences of the primers (40 μmol/L) and probes (10 μmol/L) used were as follows: FluA forward primer, 5′-GACCRATCTGTACCTCTCG-3′; FluA reverse primer, 5′-GGAGTTYGAGCACAAGCAGTGTA-3′; FluA probe, 5′-TCGAGTCTCGCTACTGGGCA-C3′; H5A forward primer, 5′-TGAAAGTGTAARAAACGGAACGT-3′; H5A reverse primer, 5′-TTGTGGCCAGYGGTACGGGACT-3′; and H5A probe, 5′-TGACTACCCCGACTTACAGAAGCAAGCAACTAA-3′.

The reaction was completed in a total volume of 25 μL, performed using the QuantiTect Probe PCR kit (Qiagen). The reaction mixture was incubated with 5 μL of RNA at the following temperature cycles. First, the reverse-transcription reaction was finished by 1 cycle at 50°C for 30 min. Next, the matrix (FluA) and hemagglutinin (H5) genes were amplified by 1 cycle at 94°C for 15 min; 45 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s each; and 1 cycle at 72°C for 5 min.

**Results and discussion.** We found that although the SA-α2,6-Gal receptor was abundantly expressed on epithelial cells throughout the GI tract, the level of SA-α2,3-Gal receptor expression gradually increased from the ileum to the rectum (Figure 1A). In the small intestine, receptors were detected only in the basal layer and in some lamina propria mononuclear cells (Figure 1A). A striking difference in receptor distribution was observed in the mucosa of the large intestine: goblet cells exclusively expressed the avianlike receptor, whereas the epithelial cells and lamina propria mononuclear cells mainly expressed the humanlike receptor (Figure 1B and 1C). Goblet cells secrete mucins, a family of large and heavily glycosylated proteins. Determination of whether binding of H5N1 viruses to goblet cells and their products protects against or enhances infection requires further investigation. Goblet cells from the upper respiratory tract exclusively expressed SA-α2,6-Gal receptors (Figure 1E), suggesting that the latter cells...
Figure 1. Distribution of sialic acid (SA) receptors in the lower gastrointestinal tract, as determined by lectin immunofluorescence. Deparaffinized sections of ileum (A), colon (B), and rectum (C) were stained with fluorescence-conjugated lectins. Red staining indicates SA-α2,3-Gal, and green fluorescence indicates SA-α2,6-Gal. White arrows indicate goblet cells from the upper respiratory tract (D) and colon (E).

may harbor the virus and provide a source from which it can spread.

We next performed ex vivo infection of human colon with a pathogenic H5N1 virus. As shown in Figure 2, the virus-specific antigen NP could be detected in human gut tissue after H5N1 virus infection (Figure 2B), whereas it could not be detected after mock infection (Figure 2A). Fresh duck gut tissue was used as a positive control (Figure 2C). Furthermore, there was increased production of viral RNA from the gut tissue 18 h after infection (Figure 2D), as determined by quantitative real-time PCR. In addition, the viral yield, as measured by the TCID₅₀ assay, in culture supernatants of the organ culture was significantly increased from 2 to 24 h after infection (mean ± standard deviation, 1517 ± 161 vs 2958 ± 168; P = .006) (Figure 2E). These results suggested that not only could the H5N1 virus directly infect human gut tissue but also that the human GI tract could support viral replication. On the other hand, we found that human colon tissues could not be infected with a seasonal influenza virus, H3N2, in parallel experiments (data not shown). The infection experiments were repeated 3 times with each virus.

Finally, for direct evidence that the H5N1 virus targets the GI tract in humans naturally infected with the virus, we stained autopsy colonic samples from a patient with a recent H5N1 case for the presence of a viral antigen of influenza A virus (NP). As shown in Figure 2F and 2G, histological examination of the colon tissue showed severe destruction of the mucosa, characterized by loss of epithelial cells and crypt distortion. Lung tissue from the same patient was used as a positive control (Figure 2H and 2I). Also, there was a diffuse, predominantly mononuclear inflammatory infiltrate in the lamina propria (Figure 2F and 2G). Furthermore, the influenza virus antigen NP was readily detected in gut tissue (Figure 2G). Virus infection in the GI tract was further confirmed by detection of viral RNA in fecal samples from 2 patients with recent H5N1 cases who had severe diarrhea (data not shown).

In this study, we have demonstrated that human gut tissues express SA receptors with α2,3 linkages, which are preferentially used by avian influenza viruses. Additional work is required to determine the precise position of the receptors, which may be expressed on goblet cells or on the protective mucins secreted by these cells. Importantly, we have presented novel data demonstrating the ability of H5N1 viruses to infect and replicate within ex vivo gut organ cultures. Although oral transmission of avian influenza virus is known to occur in a number of species, including mammals [3, 4], there is currently no direct evidence for this transmission route in humans; despite this, it cannot be ruled out.

In addition, the evidence presented here for the presence of viral antigen in the gut tissue of an infected patient supports and agrees with the findings of previous studies demonstrating the presence of viral nucleic acids and infectious virus in the GI tracts and stool of H5N1-infected individuals [2, 11, 12]. Taken together, our data suggest that the human gut is an
Figure 2. Ex vivo infection of colonic tissues with H5N1 virus. Shown is immunostaining for the nucleoprotein (NP) of influenza A virus from ex vivo organ culture of mock-infected control human tissues (A), human colonic H5N1 (A/AH/01/05) (B), and duck gut tissues infected with A/AH/01/05 (C), 24 h after infection. Two hours after infection, tissues were washed 6 times to remove unbound virus. The tissues were then cultured for another 24 h before being fixed in 10% formalin for immunohistochemical analysis. Viral load in the infected tissue explants at different time points after infection was measured by quantitative real-time polymerase chain reaction (PCR), and the results of 1 of the 4 experiments conducted are presented (D). Viral load in the supernatant from the culture of tissue explants at the corresponding time points was measured by median tissue culture infective dose (TCID 50) analysis, and the results of 1 of 4 experiments conducted are presented (E). For quantitative real-time PCR, viral load in the infected tissues, as determined by gene expression of hemagglutinin (HA), was normalized to the corresponding β-actin level in the tissues, to minimize variation due to differences in the amount of tissue between samples. Representative results of 3 independent experiments with different samples are shown. Arrows indicate the presence of NP-positive cells. Immunohistochemical staining of H5-infected colon was performed using isotype control antibody (F) and monoclonal antibody against the NP of influenza A virus (G). Arrows indicate NP-positive cells. Immunohistochemical staining of H5-infected lung was performed using isotype control (immunoglobulin G 2a) antibody (H) and monoclonal antibody against the NP of influenza A virus (I).
additional target organ for H5N1 viruses and can support avian H5N1 viral replication. As such, excretions from the GI tract of H5N1-infected individuals should be regarded as a potential source of contamination. This has considerable implications in the development of public health policy.

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References