Evidence for Influenza Virus CNS Invasion Along the Olfactory Route in an Immunocompromised Infant

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Central nervous system (CNS) disease is the most common extrarespiratory complication of influenza in humans. However, the pathogenesis, including the route of virus entry, is largely unknown. Here we present, for the first time, evidence of influenza virus entry into the CNS via the olfactory route in an immune-compromised infant. Since the nasal cavity is a primary site of influenza virus replication and is directly connected to the CNS via the olfactory nerve, these results imply that influenza virus invasion of the CNS may occur more often than previously believed.

Keywords. influenza; central nervous system; CNS disease; olfactory route; virus attachment.

Influenza virus infections are predominantly restricted to the respiratory tract, with central nervous system (CNS) disease the most common extrarespiratory complication [1]. Neurological symptoms include febrile seizures, acute brain dysfunction, Reye syndrome, (meningo)encephalitis, and encephalopathy. Influenza viruses also have been linked to Guillain–Barré syndrome, Kleine–Levin syndrome, Parkinson symptoms, and neurodegenerative diseases including postencephalitis Parkinson’s disease [2–4]. Influenza has been associated with CNS disease ever since the 1918 H1N1 pandemic [4]. During the 2009 H1N1 virus pandemic, up to 9.7% of hospitalized children had evidence of neurological complications [5]. Other influenza subtypes, including the highly pathogenic avian influenza (HPAI) H5N1 virus, also have been associated with neurological symptoms, indicating that CNS disease is not subtype restricted [1, 6, 7]. Surprisingly, the pathogenesis of influenza virus–associated CNS manifestations, including route of entry, is largely unknown.

Influenza viruses could invade the CNS via various routes, including the circulating system and cranial nerves. CNS invasion via the circulating system requires a viremic phase, which is uncommon during influenza virus infection in humans [1]. Invasion via cranial nerves has been observed in ferrets; we and others have shown that HPAI H5N1 virus entered the CNS via the olfactory route to the olfactory bulb and subsequently throughout the CNS, causing a severe meningoencephalitis [8, 9]. Furthermore, in vitro studies have shown that HPAI H5N1 virus can be transported transaxonally [10]. These studies indicate that the olfactory nerve, which directly connects the nasal cavity with the CNS, can function as a route of entry for influenza viruses. However, evidence for CNS invasion along the olfactory route in humans has never been documented, probably because appropriate tissues are rarely collected during autopsies. Here we present a case of a child with a severe immunodeficiency, in whom we found strong evidence for influenza virus spread along the olfactory route into the CNS.

METHODS

Patient Description

In 2012 an 11-month-old girl presented at the emergency ward of a secondary care hospital with fever and shortness of breath (day 1). In addition, a microcephaly (−3.6 standard deviations) was noted, and medical history revealed a delay in motor development. She had a history of sepsis without focus at the age of 5 months. Neutropenia was diagnosed for which she received sulfamethoxazole and trimethoprim for 2 months until blood neutrophil concentrations normalized. A neonatal iso- or alloimmune neutropenia was suspected at that time because of its transient nature.

On day 1, physical examination revealed bilateral crepitations over the lungs, with an oxygen saturation of 91%. Chest radiography showed an infiltrate in the right lower lung lobe that was compatible with pneumonia. A nasal swab from day 1 was negative for influenza virus, respiratory syncytial virus, and rhinovirus by polymerase chain reaction (PCR). Blood cultures on day 1 revealed no bacteria. Treatment with amoxicillin–clavulanic acid was started. The patient clinically deteriorated on day 3 and C-reactive protein increased up to 359 mg/mL, with leukocyte and thrombocyte counts of 1.0 and 8.6 × 109/L, respectively. Antibiotic treatment was switched to ceftriaxone. After further deterioration,
she was transferred to Erasmus MC on day 3 where antibiotic treatment was switched to ceftazidime and gentamicin and then changed to fluclaxacin monotherapy when blood cultures revealed *Staphylococcus aureus* bacteremia. The girl developed progressive shortness of breath with severe pulmonary crepitations and increasing infiltrates on chest radiography. Therefore, meropenem was added to the antibiotic regimen and granulocyte colony-stimulating factor was started in order to treat the severe neutropenia of 0.11 × 10⁹/L (reference >1.5 × 10⁹/L) and thymopenia of 9 × 10⁹/L (reference >206 × 10⁹/L).

On day 10, the infant was sedated and transferred to intensive care for mechanical ventilation; bronchoalveolar lavage (BAL) was performed, in which *Klebsiella pneumonia*, *Aspergillus*, *Candida albicans*, *Candida tropicalis*, and influenza A virus were detected. A new blood culture was *C. tropicalis* positive. She was treated with caspofungin, voriconazole, fluclaxacin, meropenem, and oseltamivir. In addition, she received filgrastim in order to stimulate neutrophil production. Clinical deterioration required extracorporeal membrane oxygenation, despite which she died on day 13. Post-mortem analyses revealed a severe immunodeficiency with pancytopenia characterized by 0.41 × 10⁹/L T lymphocytes (reference 1.6–6 × 10⁹/L) and 0.03 × 10⁹/L B lymphocytes (reference 0.6–2.7 × 10⁹/L). The cause of the immunodeficiency remains unknown but is likely based on either a genetic syndrome or DNA repair disorder because of the combination of severe immunodeficiency with microcephaly and neurodevelopmental delay. Informed consent was obtained from the patient’s caretakers to use postmortem tissues for diagnostic and research purposes.

**Analysis of Patient Materials**

During autopsy, samples were collected from the respiratory tract (trachea, bronchi, and lung), heart, thymus, thyroid, lymph nodes, spleen, liver, gut, kidney, urinary bladder, ovaries, and skeletal muscle. Olfactory bulb, cerebrum, cerebellum, spinal cord, and pituitary samples were collected from the CNS. During autopsy, samples were collected from the respiratory tract (trachea, bronchi, and lung), heart, thymus, thyroid, lymph nodes, spleen, liver, gut, kidney, urinary bladder, ovaries, and skeletal muscle. Olfactory bulb, cerebrum, cerebellum, spinal cord, and pituitary samples were collected from the CNS. After formalin fixation and paraffin embedding, hematoxylin and eosin stained sections were screened for histological lesions. Tissue sections were screened for influenza virus antigen using immunohistochemistry [11]. RNA was extracted from paraffin sections using an RNeasy FFPE kit (Qiagen, Hamburg, Germany) and screened for influenza RNA by reverse-transcription polymerase chain reaction (RT-PCR) [12]. In addition, RNA was extracted from the BAL and plasma from day 12 in order to detect viral RNA by RT-PCR [12] and to perform hemagglutinin and neuraminidase sequence analyses. Virus culture was attempted by coculturing BAL, trachea, and lung tissues with Madin-Darby canine kidney (MDCK) or MDCK-β-Galactoside α-2,6-Sialytransferase (SIAT1) cells.

**Attachment of Different Influenza Viruses to Olfactory Mucosa**

We have shown that the ability to attach to host cells is an important determinant for the cell tropism of influenza viruses. Therefore, the ability of seasonal H3N2 viruses from 2012 (A/Netherlands/752/12 and A/Netherlands/1251/12) to attach to human olfactory mucosa was determined by virus histochemistry [13]. For comparison, we included seasonal H3N2 virus from 2003 (A/Netherlands/212/03), 2 HPAI H5N1 viruses (A/Vietnam/1194/04 and A/Indonesia/05/05), and 1 pandemic H1N1 virus (A/Netherlands/602/09). Normal human olfactory mucosa (n = 3) was obtained via The Netherlands brain bank, Netherlands Institute for Neuroscience in Amsterdam, which is a non-profit organization that collects human brain tissues of donors, and the Department of Pathology, Erasmus MC. All materials were collected from donors who provided written informed consent for a brain autopsy and the use of material for research purposes. In addition, ferret olfactory mucosa (n = 2) was included since the ferret serves as a model for influenza virus–associated CNS disease [8]. Olfactory mucosa samples were stained for neuron-specific enolase (NSE) to confirm neuronal origin.

**RESULTS**

Influenza virus antigen could only be detected in the olfactory bulb, olfactory tract, and gyrus rectus, which is adjacent to the olfactory bulb. Virus antigen was detected in both neurons and glial cells based on morphology (Figure 1). No influenza virus antigen was detected in any other part of the CNS, respiratory tract, or other organs.

Gross pathology revealed no abnormalities around the olfactory nerve or cribriform plate. The brain was microcephalic with normal cellularity of the arachnoidal tissue. Structural changes in the CNS were diffuse cortical polymicrogyria, most prominent at the level of the insula. In the white matter, there was evidence of perivascular psammomatous mineralizations. No lesions or vascular abnormalities were associated with the presence of virus antigen. The lungs showed diffuse hemorrhages, congestion, few giant cells, many macrophages, and multifocal organizing pneumonia, with histological evidence of both bacterial and fungal colonization. All solid organs within the thorax showed petechiae. Lymph nodes were small or undetectable, except for those draining trachea and lung. Lymph nodes and thymus were hypoplastic. The liver showed micro- and macrovesicular steatosis.

Sequence analyses of RNA extracted from the BAL confirmed the presence of a seasonal influenza H3N2 virus. Subsequent hemagglutinin- and neuraminidase-specific RT-PCR confirmed the presence of the same subtype in pooled RNA from the olfactory bulb and gyrus rectus. Unfortunately, due to the limited amount of tissue available and the poor quality of RNA rescued from the paraffin-embedded tissues, it was not possible to confirm that the viruses detected in the BAL and CNS were genetically identical. Viral RNA could not be detected in a plasma sample from day 12, excluding a viremia, or in RNA extracted from
formalin-fixed tissues from other parts of the CNS and respiratory tract. No virus was cultured from BAL or respiratory tissues.

Both seasonal H3N2 viruses isolated in 2012 attached abundantly to the apical side of human olfactory mucosa (Figure 2). Seasonal H3N2 virus from 2003, pandemic H1N1, and both HPAI H5N1 viruses attached to the apical side of human olfactory mucosa. All viruses had a similar pattern of attachment to ferret and human olfactory mucosa (Figure 2).

DISCUSSION

Here, we present, for the first time, evidence that a seasonal H3N2 virus may have entered the CNS via the olfactory route in an immunocompromised infant. First, the restricted localization of influenza virus in the olfactory bulb and gyrus rectus corresponded to that seen in the early stage of HPAI H5N1 virus spread from the nasal cavity to the CNS via the olfactory route in ferrets [8]. Second, influenza virus was not detected in any other part of the CNS or in plasma samples, making virus entry via the circulatory system less likely.

The ability of 2 seasonal H3N2 viruses from 2012 to attach to the apical side of normal human olfactory mucosa supports the idea that this virus used the olfactory tract to spread from the nasal cavity to the CNS. Unfortunately, olfactory mucosa was not collected at autopsy, and we could not determine whether there was infection of olfactory receptor neurons (ORNs). ORNs have a dendrite that extends in the nasal cavity lumen and an axon that penetrates the cribriform plate and extends to the olfactory bulb where it has contact with neuronal and glial cells. After infection of these ORNs, influenza virus could be transported transaxonally toward the olfactory bulb [10]. In addition to H3N2 isolates from 2012, a 2003 isolate, pandemic H1N1, and HPAI H5N1 viruses all attached to olfactory mucosa, suggesting that this property is not a unique or novel feature of recent seasonal H3N2 viruses. This suggests that ORNs might be susceptible to multiple influenza virus subtypes. Seasonal H3N2, pandemic H1N1, and HPAI H5N1 viruses also attached to ferret olfactory mucosa, which corresponds to the infection of ORN in ferrets inoculated with seasonal H3N2 virus, pandemic H1N1, and HPAI H5N1 viruses [14].

It is not known whether the severe immunodeficiency—with low levels of both T cells and B cells—from which this child was suffering, predisposed to influenza virus entry via the olfactory route. Immunodeficiency is known to predispose other viruses, for example, herpesvirus and adenovirus, to CNS infections. It should be realized that since this infant was suffering from an unknown immunodeficiency, an extensive autopsy was performed, which resulted in the observations described here. In general, autopsies in which the CNS is extensively sampled are rarely performed.

There was no evidence of neurological signs in this child, which corresponds with the restricted localization of influenza virus in the CNS and the absence of associated histological lesions. However, neurological signs may have been masked due to sedation. The clinical impact of CNS invasion via the olfactory nerve could vary from decreased consciousness to development of a severe encephalitis [1, 2]. In addition, entry of pathogens along the olfactory nerve might cause damage that accumulates over time, contributing to the development of neurodegenerative diseases [15]. It is known that seasonal influenza virus infections can cause Parkinson-like symptoms, and
invasion of HPAI H5N1 virus in the CNS of experimental animals is known to induce local immune responses, increased phosphorylation, and aggregation of alpha synuclein, which are hallmarks of neurodegenerative disorders in experimental animal models [9, 10].

The frequency of influenza virus entry into the CNS via the olfactory route and associated disease manifestations remain unknown, and it is not known whether this could occur in immunocompetent individuals. Influenza virus entry via the olfactory nerve might occur more frequently since ORNs are located

Figure 2. Influenza virus attachment to human and ferret olfactory mucosa. A specific immunohistochemical staining for neuron-specific enolase (NSE) stains the neuronal cells in the olfactory mucosa and nerve twigs in the submucosa (top panel). Seasonal H3N2, pandemic H1N1, and HPAI H5N1 viruses attached to the apical side of human and ferret olfactory mucosa.
in the nasal cavity, which is the primary replication site for most influenza viruses. CNS invasion and associated disease caused by influenza viruses might thus not only be a complication of a severe lower respiratory tract infection. This would fit with case reports of influenza–associated encephalitis without initial lower respiratory tract disease [6, 7].

Taken together, this study provides evidence that a seasonal H3N2 influenza virus used the olfactory route to invade the CNS in a severely immunocompromised infant. To obtain more insight into the frequency and clinical impact of CNS invasion via the olfactory nerve, we emphasize that in any autopsy case involving influenza, the olfactory tract should be examined for evidence of local virus replication.

Notes

Acknowledgments. We thank Anna van der Linden and Jaap Bongers for excellent technical assistance.

Financial support. This work was supported by European Union FP7 ANTIGONE (278976) and the Netherlands Organisation for Scientific Research (NWO, 91614115).

Potential conflicts of interest. A. D. M. E. O. is partly employed by ViroClinics Biosciences B.V. and owns share certificates in ViroClinics Biosciences B.V. T. K. is a part-time consultant for ViroClinics Biosciences B.V. Other authors have no conflict of interest. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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