

So Many Questions, So Little Time

Ruben O. Donis and Nancy J. Cox

Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Fifteen years have elapsed since the first human infections with H5N1 highly pathogenic avian influenza (HPAI) virus were detected in Hong Kong SAR, China [1, 2]. Many of these human infections were fatal and represented the first documented outbreak of severe disease due to HPAI viruses. The hallmark of these H5N1 viruses is their hemagglutinin genes inherited from A/goose/Guangdong/1996, a virus first detected during an outbreak in domestic geese in China. Who could have predicted the current H5N1 situation in 1997? As of August 2012, H5N1 viruses of this lineage have caused 607 reported human cases (358 fatal), the culling of 400 million birds, and have become entrenched in poultry populations on 2 continents [3, 4]. Early expectations were that this virus would be eliminated quickly and become extinct after local bird populations were decimated by the outbreak or culled, thus recapitulating the experience with dozens of HPAI outbreaks in poultry in many countries during the past century. Rather than becoming extinct, these H5N1 viruses spread to 60 countries on 3 continents by 2006 [3]. The alarm caused by the

far-ranging geographic expansion of H5N1 galvanized governments to invest resources in avian disease control. Despite many effective local and regional infection control programs in recent years, H5N1 is considered to be endemic in poultry in many countries, including Indonesia, Bangladesh, China, Vietnam, and Egypt [3]. Several of these countries also report the highest numbers of human infection. Most HPAI H5N1 human infections reported to the World Health Organization by public health authorities since 1997 were acquired by direct or indirect contact with infected birds.

This spread and entrenchment of H5N1 has sparked a lively debate concerning the likelihood and severity of an H5N1 pandemic. However, recent studies conducted in the United States and The Netherlands [5, 6] demonstrated that H5N1 can gain airborne transmissibility for ferrets after acquiring a relatively small number of genetic changes (reassortment and/or mutations). Additional studies [7] indicated that the pandemic potential of H5N1 viruses currently endemic in Asia and Africa is no longer a remote theoretical risk, prompting calls for increasing the pace of research to control H5N1 infection in poultry to pre-empt a future pandemic. However, international animal health experts agree that global H5N1 eradication from poultry may take several decades [8]. Therefore, many critically important questions need to be answered in a race against the pandemic

clock. Furthermore, these research questions should be translated into improved virus detection and intervention methods that can be applied globally to control influenza.

The severity of a potential H5N1 pandemic is of critical importance for pandemic planning. The case-fatality rate of H5N1 infection has been difficult to establish with any certainty, mainly because current surveillance methods are likely to miss milder infections [9, 10]. Nevertheless, the potential lethality of H5N1 infections in healthy persons of all ages is undeniable, and pathogenesis observed in critically ill patients appears to be linked to the so-called “cytokine storm,” or hypercytokinemia [11–13]. The molecular basis for hypercytokinemia has puzzled scientists and clinicians for many years. An article in this issue of *the Journal of Infectious Diseases* (Sakabe et al, doi:10.1093/infdis/jis523) and a recent article in *Science* [14] provide important clues. Both articles identify one of the viral polymerase complex genes as a determinant of the cytokine responses in human cell cultures and in mouse models.

The research reported in this issue by Sakabe et al relied on reverse genetics to elucidate the molecular basis for striking differences between H5N1 viruses in their ability to activate macrophage cytokine secretion, which the authors observed in earlier studies [15]. A panel of reassortant viruses was created by using 2 human H5N1 viruses with

Received 13 August 2012; accepted 23 August 2012.

Correspondence: Nancy J. Cox, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd, MS-A20, Atlanta, GA 30333 (njc1@cdc.gov).

The Journal of Infectious Diseases

Published by Oxford University Press on behalf of the Infectious Diseases Society of America 2012.

DOI: 10.1093/infdis/jis529

differential induction of cytokine secretion, a high cytokine inducer A/Vietnam/UT3028II/03 clone 2 (VN3028IIc2) and a low inducer A/Indonesia/UT3006/05 (IDN3006). A systematic analysis of this panel of reassortant viruses revealed that the PA gene from VN3028IIc2 was correlated with strong induction of IL-6, TNF α , CCL3, CCL4, CCL5, CXCL9, and CXCL10 secretion in infected human monocyte-derived macrophages and that reassortants with this gene tended to be more virulent in the mouse model. The differential transcriptional up-regulation of secreted cytokines was confirmed by microarray analyses, which also revealed an increased expression of genes related to cytokine signaling. In addition, pathway analyses identified a network of differentially expressed genes related to increased cytokine signaling in macrophages infected with viruses containing the PA gene from the high cytokine inducer VN3028IIc2, compared with those infected with IDN3006. These findings have important implications for risk assessment of H5N1 viruses occurring in nature and may also inform development of improved live attenuated influenza vaccines. The modulation of cytokine responses by influenza viruses has often been associated with their virulence in animal models and linked to human disease [16–20]. Although the influenza NS1 protein has been deemed the master viral regulator of host innate immunity, it is becoming increasingly clear that the virus has built-in redundancy for this critically important function [16, 18, 19, 21–23]. While this article was in press, an article was published in *Science* [14] indicating that the PA gene of influenza A encodes a new protein, termed PA-X, by ribosomal frame shifting. This novel influenza protein comprises the endonuclease domain of the PA protein joined to a C-terminal domain encoded by the newly discovered ORF and functions to repress cellular gene expression. PA-X decreased the pathogenicity of the reverse genetics-derived 1918 pandemic influenza virus

by modulating the inflammatory, apoptotic, and T lymphocyte signaling pathways. In both articles, the authors noted that the viruses with differential cytokine regulatory properties replicated with nearly identical efficiency in vitro and in vivo (ie, differences in cytokine responses were not simply mirroring changes in viral replication or gene expression levels). It will be interesting to analyze the PA gene sequences of the high and low cytokine inducer H5N1 viruses reported by Sakabe et al, to determine whether polymorphisms in the expression of the novel PA-X are associated with the observed differential regulation of cytokine production.

Another commonality between these 2 articles is the experimental approach (ie, the use of reverse genetics techniques to construct H5N1 or 1918 H1N1 viruses with enhanced virulence, compared with a control, to demonstrate that specific viral genes modulate virulence). Both studies were evaluated as described in the policy for oversight of life sciences Dual Use Research of Concern (DURC) issued by the US Government [24]. Both articles identifying a role for the PA gene in influenza virus pathogenesis were subjected to institutional biosecurity review processes and published, in the best interest of public health and national security. Publication of important research on the role of the PA protein in influenza virus pathogenesis is essential to our understanding of virus-host interactions and provides insight that can be used to develop effective countermeasures for severe systemic disease involving hypercytokinemia. Nevertheless, uncertainty about publication of research on H5N1 that could be perceived as having dual use potential may become a deterrent for institutions and investigators to conduct influenza research that is essential for pandemic preparedness.

These important studies could not have been done without influenza surveillance in humans and animals. Although data and viruses collected during

influenza surveillance are critically important for research such as this and for pandemic preparedness, this information is insufficient to fully assess the risk of pandemic emergence. Although it is clear that surveillance for H5N1 in both animals and humans needs to be improved, it is also clear that the effectiveness of surveillance is greatly enhanced by a predictive understanding of the molecular characteristics of viruses that have the greatest propensity for zoonotic transmission. Blunting the impact of a future pandemic will require interventions based on a solid understanding of the transmission dynamics of emerging pandemic viruses and the molecular markers of viral transmissibility and pathogenicity. Scientific and technological innovation to achieve influenza public health preparedness goals is based on a combination of conceptual creativity and new tools to improve early outbreak detection and evidence-based implementation of validated influenza control. Although development of universal vaccines that elicit full and long-lasting protection and are readily scalable for rapid mass production in emergency situations would provide the strongest countermeasure for pandemic mitigation, we are a long way from achieving this goal. In the meantime, what needs to be done to safeguard the execution of these vitally important studies? How can we ensure that essential discoveries, such as the role of the PA gene in modulating host cytokine production and pathogenesis, as described by Sakabe et al in this issue, will continue to support the global pandemic preparedness objectives?

A concerted global effort is necessary to answer these questions. The public, along with members of the scientific, public health, agricultural, and biosecurity communities, each with their different perspectives and concerns, must engage in a thoughtful dialogue concerning the benefits and risks of gain of function studies on H5N1 viruses. When consensus is developed, broad multi-

sectorial engagement will be needed to ensure the commitment of human and animal health resources to develop the scientific knowledge that will serve as the basis for effective interventions against the global threat of H5N1. This challenge clearly transcends pandemic influenza, and our response to conducting research on H5N1 viruses may well become a paradigm that applies to other areas of biological research. The health and well-being of this and future generations are at stake. With so many questions to answer and H5N1 viruses in constant evolution, we cannot afford to postpone broad stakeholder engagement.

Notes

Acknowledgments. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the views of the funding sources or the Centers for Disease Control and Prevention.

Potential conflicts of interest. All authors: No reported 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.

References

1. Claas EC, Osterhaus AD, van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **1998**; 351:472–7.
2. Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **1998**; 279:393–6.
3. FAO-DAH. H5N1 HPAI Global Overview. January–March **2012** <http://www.fao.org/docrep/015/an388e/an388e.pdf>. Accessed July 24 2012.
4. WHO-CSR. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. (6 July), **2012**. Available at: http://www.who.int/influenza/human_animal_interface/EN_GIP_20120706CumulativeNumberH5N1cases.pdf. Access July 23 2012.
5. Herfst S, Schrauwen EJ, Linster M, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **2012**; 336:1534–41.
6. Imai M, Watanabe T, Hatta M, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **2012**; 486:420–8.
7. Russell CA, Fonville JM, Brown AE, et al. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* **2012**; 336:1541–7.
8. FAO-DAH. Stamping out H5N1 avian influenza could take decades, **2011**. <http://www.fao.org/news/story/en/item/66118/icode/>. Accessed July 23 2012.
9. Osterholm MT, Kelley NS. Mammalian-transmissible H5N1 influenza: facts and perspective. *MBio* **2012**; 3:e00045.
10. Wang TT, Parides MK, Palese P. Seroevidence for H5N1 influenza infections in humans: meta-analysis. *Science* **2012**; 335:1463.
11. de Jong MD, Simmons CP, Thanh TT, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* **2006**; 12:1203–7.
12. Peiris JS, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* **2004**; 363:617–9.
13. Cheung CY, Poon LL, Lau AS, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* **2002**; 360:1831–7.
14. Jagger BW, Wise HM, Kash JC, et al. An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response. *Science* **2012**; 337:199–204.
15. Sakabe S, Iwatsuki-Horimoto K, Takano R, et al. Cytokine production by primary human macrophages infected with highly pathogenic H5N1 or pandemic H1N1 2009 influenza viruses. *J Gen Virol* **2011**; 92:1428–34.
16. Spesock A, Malur M, Hossain MJ, et al. The virulence of 1997 H5N1 influenza viruses in the mouse model is increased by correcting a defect in their NS1 proteins. *J Virol* **2011**; 85:7048–58.
17. Belser JA, Wadford DA, Pappas C, et al. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. *J Virol* **2010**; 84:4194–203.
18. Wolff T, Ludwig S. Influenza viruses control the vertebrate type I interferon system: factors, mechanisms, and consequences. *J Interferon Cytokine Res* **2009**; 29:549–57.
19. Fernandez-Sesma A, Marukian S, Ebersole BJ, et al. Influenza virus evades innate and adaptive immunity via the NS1 protein. *J Virol* **2006**; 80:6295–304.
20. Kash JC, Tumpey TM, Proll SC, et al. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **2006**; 443:578–81.
21. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, et al. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. *Proc Natl Acad Sci USA* **2009**; 106:3455–60.
22. Billharz R, Zeng H, Proll SC, et al. The NS1 protein of the 1918 pandemic influenza virus blocks host interferon and lipid metabolism pathways. *J Virol* **2009**; 83:10557–70.
23. Jackson D, Hossain MJ, Hickman D, Perez DRLamb RA. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. *Proc Natl Acad Sci USA* **2008**; 105:4381–6.
24. Office of Biotechnology Activities N.I.H. United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern, **2012**. Available at: http://oba.od.nih.gov/oba/biosecurity/pdf/united_states_government_policy_for_oversight_of_durc_final_version_032812.pdf. Accessed July 22 2012.