Influenza Viruses: Breaking All the Rules

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ABSTRACT Influenza A viruses (IAV) are significant pathogens able to repeatedly switch hosts to infect multiple avian and mammalian species, including humans. The unpredictability of IAV evolution and interspecies movement creates continual public health challenges, such as the emergence of the 2009 pandemic H1N1 virus from swine, as well as pandemic threats from the ongoing H5N1 and the recent H7N9 epizootics. In the last decade there has been increased concern about the "dual use" nature of microbiology, and a set of guidelines covering "dual use research of concern" includes seven categories of potentially problematic scientific experiments. In this Perspective, we consider how in nature IAV continually undergo "dual use experiments" as a matter of evolution and selection, and we conclude that studying these properties of IAV is critical for mitigating and preventing future epidemics and pandemics.

Influenza A viruses (IAV) are important pathogens of humans and animals that cause continually recurring epizootics, annual epidemics, and periodic pandemics. As single-stranded segmented RNA viruses of the family *Orthomyxoviridae*, IAV show antigenic diversity that reflects 17 different hemagglutinin (HA) and ten neuraminidase (NA) surface protein subtypes. Multiple HA-NA subtype combinations and genotypes are generated through mixed infection and reassortment, and an error-prone RNA-dependent RNA polymerase generates complex viral quasispecies, from which variants can be rapidly selected under such evolutionary pressures as exposure to new host species, host immunity, and antiviral drugs. IAV host barriers are relatively weak, allowing viruses to repeatedly switch hosts to infect multiple avian and mammalian species. The unpredictability of IAV evolution and interspecies movement creates continual public health challenges (1, 2).

All avian and mammalian IAV are believed to have descended from avian influenza viruses resident in an enormous global avian virus gene pool. These viruses infect hundreds of wild bird species and undergo frequent reassortment (3). Occasionally, viruses from this avian pool switch hosts to infect either domestic poultry or mammals. The mechanisms of host switching and stable adaptation to new host populations are incompletely understood (4). In 1997, highly pathogenic avian influenza A viruses (HPAI) of the H5N1 subtype in Asia escaped the wild avian gene pool and became adapted to domestic poultry. These H5N1 viruses have circulated enzootically in domestic poultry for over 15 years, spreading to the Middle East, Africa, and Europe (5, 6). Spillover from poultry to humans has caused 630 documented human infections with 375 fatalities (as of 4 June 2013; WHO) (7), without adaptation to or enhanced transmissibility between humans. (It should be noted that poultry-adapted IAV rarely infect humans and that the underlying basis and mechanisms of these rare but severe cases of human H5N1 infections are unknown). More recently (starting in February 2013), a novel H7N9 virus, presumably also from a domestic poultry source, has caused 132 documented human infections with 37 fatalities (as of 7 June 2013) (8). Whether this virus will adapt to efficient human-to-human transmissibility or infect other mammals is unknown (9–11).

Over the last decade, there has been increased concern about the "dual use" nature of microbiology, including concerns about misapplication of scientific investigation for bioterrorism or bioweapons development and concerns about accidental release of consequential pathogens from laboratories in which they are being studied or stored. Recent experiments with strains of HPAI H5N1 engineered to enhance ferret and guinea pig transmissibility have raised additional biosafety concerns (12–17). As a result, in 2012, the U.S. government established policies for the oversight of "dual use research of concern" (DURC) relating to fifteen "high-consequence" pathogens and toxins, two of which are IAV: HPAI H5N1 and the reconstructed 1918 pandemic influenza virus (18). DURC was defined as "life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security" (18). DURC policy (here referred to as "dual use" policy) is defined as including seven categories of potentially problematic scientific experiments as follows:

(i) enhancing the harmful consequences of an agent or toxin

(ii) disrupting immunity or the effectiveness of an immunization against an agent or toxin without clinical or agricultural justification

(iii) conferring to an agent or toxin resistance to clinically or agriculturally useful prophylactic or therapeutic interventions against the agent or toxin, or facilitating its ability to evade detection methodologies

(iv) increasing the stability or transmissibility of or the ability to disseminate an agent or toxin

(v) altering the host range or tropism of an agent or toxin

(vi) enhancing the susceptibility of a host population to an agent or toxin

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(vii) generating or reconstituting an eradicated or extinct agent or toxin

In this Perspective, we consider how in nature IAV continually undergo such "dual use experiments" as a matter of evolution and selection and how such viral evolution determines the consequent capacity of IAV to cause harm. These considerations lead us to conclude that scientifically studying such fundamental evolutionary properties of IAV is critical for mitigating and preventing the devastation these viruses cause. We do not imply that all IAV evolution in nature should be considered dual use but rather hope that consideration of the dynamic nature of IAV biology will inform policy decisions.

Naturally occurring IAV routinely break the enumerated dual use rules. (i) Enhancing the harmful consequences of an agent. The ability of different strains of IAV to cause disease varies widely between viruses and infected hosts. Numerous examples exist in which IAV strains emerge in nature with enhanced pathogenicity, transmissibility, or other altered phenotypic properties.

The emergence of HPAI strains in domestic poultry is one such example. These viruses share a class of mutation, independently arising on numerous occasions, creating an insertional element in the HA cleavage site of avian H5 or H7 subtype IAV which codes for multiple basic amino acids (19) and which allows these viruses to be activated by ubiquitous furin-like proteases. Such insertions create strains capable of causing systemic viral replication associated with severe and fatal disease in infected poultry (i.e., HPAI strains) but generally not in humans or other mammals, in which HPAI viruses as a group are unable to cause disease. In the case of HPAI H5N1, epizootic poultry disease also forms a bridge of viral access to poultry-exposed humans.

Other noteworthy examples of IAV breaking this first dual use rule include the emergence of pandemic IAV and the continual evolution of seasonal IAV strains. IAV pandemics have occurred sporadically since at least 1510 (20) and four times in the last 100 years—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1 (21). Each pandemic emergence has featured an antigenically novel HA associated with enhanced morbidity and mortality (22), including those in younger age groups (23). The epidemiological impact of seasonal influenza varies from year to year (24), with morbidity and mortality being in some years on par with those of pandemic years (21). For example, the moderately severe A/Fujian/411/2002-like epidemic of the 2003-to-2004 season (25) was caused by an intrasubtypic reassortment between different clades of human H3N2 viruses (26, 27). It is sobering to realize that virtually all known deaths from influenza—23,000 to 36,000 deaths in the United States in a typical year and as many as 50 million deaths globally during a pandemic—result from the natural ability of IAV to break this first dual use rule by undergoing genetic alterations in nature that enable efficient infection, severe disease, and sometimes death.

(ii) Disrupting immunity or the effectiveness of an immunization. Like other RNA viruses, IAV disrupt host immunity by a variety of mechanisms, including down-regulating host type I interferon (IFN) responses (28). One key component of this is the IAV nonstructural protein NS1, which acts pleiotropically to attenuate the host interferon responses. Sequence variations, mutations, and in-frame deletions within this gene among different IAV strains may account for altered pathogenicity (29). For example, the NS1 protein from the 1918 influenza virus is a very potent inhibitor of antiviral and type I IFN responses in cultured human lung cells (30). Other NS1 mutations have been correlated with enhanced pathogenicity of HPAI H5N1 viruses (31–33). The alternatively spliced PB1-F2 may independently alter host IFN signaling via a mitochondrial component of the pathway (34), and mutations in the PB1-F2 protein of the 1918 virus and some HPAI H5N1 viruses have been linked to enhanced pathogenicity (35) and enhanced risk of secondary bacterial coinfections (36, 37). The newly described frameshifted PA-X protein may also play a role in host response modulation: reduced PA-X expression augments the pathogenicity of the 1918 influenza virus in mice (38).

One of the greatest hurdles to mitigating the public health impact of influenza is the rapid and constant evolution of seasonal IAV (39), necessitating the annual production of vaccines containing new strains that keep pace with the antigenic drift of circulating strains (40). Regular emergence of antigenic escape mutants in the HA and also in the neuraminidase (NA) genes is a defining feature of IAV biology in humans (41, 42) and an important example of dual use rule-breaking in nature. Antigenic escape occurs through acquisition of point mutations in the HA and NA genes, by intrasubtypic reassortment, or, dramatically, by the *de novo* emergence of pandemic virus strains with novel HA subtypes. If human IAV were unable to undergo continual and successful antigenic drift, growing population immunity would soon drive them to complete extinction. Breaking the second dual use rule is thus a fundamental mechanism of human influenza disease pathogenesis.

(iii) Conferring resistance to prophylactic or therapeutic interventions. Four approved antiviral drugs in two categories—the adamantane ion channel blockers and the neuraminidase inhibitors—are available for IAV treatment. Unfortunately, human and animal origin IAV often develop mutations conferring resistance to each of these drugs (43). Circulating human seasonal H3N2 viruses developed adamantane resistance after 2004 (44), severely limiting drug utility in prevention and treatment. Similarly, circulating human seasonal H1N1 viruses developed resistance to the most commonly used neuraminidase inhibitor, oseltamivir (45, 46). Upon its introduction in humans, 2009 pandemic H1N1 (H1N1pdm) viruses were already resistant to the adamantanes by virtue of a mutation in the matrix M2 gene derived from one of the parental swine IAV (47) involved in the reassortment event that generated it (48). Isolates with resistance to oseltamivir were also commonly observed (49). Resistance to other neuraminidase inhibitors, including zanamavir and the newer peramivir, has also been described with different subtypes of human IAV (50–53), and oseltamivir resistance has been described after treatment of patients infected with HPAI H5N1 viruses (54, 55). The recent H7N9 viruses possess an M2 mutation conferring adamantine resistance (56), and one of the human isolates has an NA mutation that likely confers reduced sensitivity to oseltamivir (57). Nature therefore easily and repeatedly breaks the third dual use rule by creating drug-resistant mutant viruses, reducing IAV treatment options.

(iv) Increasing the transmissibility of the agent. Pandemic IAV strains are zoonotically derived, either through reassortment with human- or mammal-adapted influenza virus or through *de novo* adaptation. The 2009 pandemic H1N1 virus was derived by reassortment of two preexisting swine IAV (48), which had both circulated in pigs and had caused only limited dead-end infections in humans (58). Why then did the 2009 pandemic reassortant

virus transmit between humans? Recent studies have shown correlations between transmission in ferrets and the unique constellation of genes associated with the 2009 pandemic virus (the Eurasian avian-like swine H1N1 IAV parental virus contributed its NA and matrix segments), in which both NA activity and viral morphology played a role in enhanced respiratory droplet transmission (59) as well as HA-NA functional balance (60). Since all known pandemic influenza viruses have been derived from precursor zoonotic IAVs or their gene segments and yet are necessarily highly transmissible between humans, new pandemic viruses must acquire genetic changes that break the fourth dual use rule of enhancing transmissibility; moreover, because pandemic viruses give rise to subsequent seasonal IAV, all human IAV result directly from the continual breaking of this rule.

(v) Altering the host range of the agent. One of the most important features of IAV ecobiology is the ability to undergo stable host switching, including bird-to-mammal switches (2, 61). The host range of IAV is very broad, including many species of wild birds, domestic anseriform and gallinaceous poultry, humans, swine, horses, dogs, cats, seals, and other mammals (2). All pandemic IAV infections are likely zoonotic, involving adaptations to humans of zoonotically derived viruses either *in toto* (e.g., 2009 pH1N1 [48] and possibly 1918 H1N1 [62]) or by reassortment that incorporates avian influenza virus gene segments into an existing human-adapted virus (e.g., 1957 H2N2 and 1968 H3N2 [63, 64]). Numerous other alterations of host range have been documented, some of which are noted here.

In 1979, an avian H1N1 virus adapted *in toto* to northern European swine (65, 66), creating a new swine lineage genetically and antigenically distinct from the North American classical swine H1N1 lineage thought to be derived from the 1918 pandemic influenza virus (67, 68). This new H1N1 lineage then replaced classical swine H1N1 viruses in Europe (69). Other avian IAV have also infected swine, including an independently emerging avian H1N1 virus in China (70). H4N6, H3N3, H1N1, and H2N3 avian viruses have all caused swine epizootics in North America (71–73).

Reassortment between swine IAV strains resulted in the 2009 pandemic H1N1 emergence, a particularly unfortunate example of many such swine host-switching events that have been occurring with increasing frequency (74). One of the parental viruses of the 2009 pandemic was derived from a North American lineage of swine-adapted IAV, itself a product of complex reassortment events between swine, human, and avian IAV (75). These triple-reassortant swine viruses, containing an epizootically active "triple-reassortant internal gene," or TRIG, cassette, have been evolving dynamically at the swine-human interface (76–78), and human infections with a novel variant H3N2 swine IAV have recently been noted as well (79, 80). The recent development of complex global hyperevolution of IAV within swine is an ominous occurrence that may greatly increase the risk of future pandemics.

Equine influenza has been recognized clinically for centuries (81) and since the 1950s has been linked separately to H7N7 and H3N8 viruses. In 1989, an independent avian H3N8 host-switch event caused a serious new equine epizootic in China (82). Interestingly, equine H3N8 viruses have undergone further host-switching events to become stably adapted to and transmitted between dogs (83). Clinically documented for centuries, canine influenza has long been considered a dead-end infection acquired from horses which never led to stable canine adaptation. Yet an avian H3N2 virus recently jumped into dog populations in North Korea and China, creating a second new stably adapted canine virus (84). Curiously, these two canine-adapted IAV strains, as well as a group of new swine H3N2 viruses with the TRIG cassette and the related 2009 pandemic H1N1 virus, all have the same peculiar genetic marker in the PA-X open reading frame that may indicate an unappreciated property of host switching or new-host adaptation (85). Thus, IAV exist in a large complex ecosystem that includes not only hundreds of species of wild birds but also poultry and numerous mammals and is characterized by numerous naturally occurring host-switching events that break the fifth dual use rule. Understanding how these host switches occur is an important and growing area of research likely to have a bearing on understanding and controlling pandemic emergence.

(vi) Enhancing the susceptibility of a host population. Enhancing the susceptibility of a host population can theoretically occur via a number of different mechanisms, including several of those already discussed, such as disrupting immunity or eluding immune response to immunizations, conferring resistance to prophylactic agents, improving transmissibility, and altering host range. In addition, influenza viruses may break the susceptibility rule by enhancing susceptibility to copathogenic infectious diseases. It is clearly established that secondary bacterial pneumonias following primary influenza virus infection play a key role in enhanced IAV morbidity and mortality (86, 87). Nasopharyngeal carriage of potential respiratory bacterial pathogens is common (88–91) and may predispose to coinfection (86). The pathogenetic mechanisms by which influenza virus and bacterial coinfection induce more-severe disease are complex and multifactorial (86, 92, 93) and include not only poorly understood viral properties but also respiratory epithelial dysfunction, impaired mucociliary clearance, enhanced bacterial adhesion, epithelial cell death, and apoptosis. Some IAV (e.g., the 1918 pandemic virus) are more capable of enhancing susceptibility to secondary bacterial pneumonia, albeit by mechanisms not fully understood. That nature alters the susceptibility of humans to severe and fatal pneumonias caused by viruses that interact with other microorganisms in the production of copathogenetic disease is another cogent example of breaking this sixth dual use rule.

(vii) Generating or reconstituting an eradicated or extinct agent. The 1918 pandemic virus was sequenced and reconstructed using an archaevirologic approach (94); study of this virus has yielded many insights into IAV biology (95). Epidemiological data, however, also support the idea of cyclic reemergence of human pandemic IAV with antigenically related HAs (i.e., antigenic recycling), leading to relative protection of persons of certain age groups in different pandemics (20). For example, elderly populations in 1918 are thought to have experienced immunoprotection derived from prior exposure to an antigenically related virus which may have emerged in the 1830s (20, 96); serologic and epidemiological data from the 1968 pandemic suggest analogous immunoprotection in persons born before 1893 (97), supporting the idea that the 1889 pandemic could have been caused by an H3 subtype virus. The 2009 pandemic virus has an H1 HA antigenically related to, and in fact directly derived from, the 1918 virus via the classical swine H1N1 viral lineage, explaining why persons born before 1950 showed evidence of immunoprotection in 2009 (98). Since HA subtypes circulating in the wild bird reservoir undergo little directed antigenic drift (3), independently emerging pandemic viruses bearing the same HA subtype are likely to be

antigenically similar. Thus, nature continues to repeatedly break this final dual use rule by recycling viruses or viral antigens from the past.

CONCLUSION

Influenza A viruses are important human and animal pathogens and are among the leading infectious causes of human deaths globally. Influenza pandemics have been emerging for over a millennium, and we will undoubtedly see novel IAV continue to evolve for efficient human adaptation and pandemic spread in the future. Whether currently circulating avian H5N1, H9N2, or the more recent H7N9 viruses can adapt to efficient transmissibility in humans remains unknown, but the consequences of such a host switch event might be devastating (14). It is of vital importance to continue to study, utilizing appropriate biosafety and biosecurity oversight, how IAV can switch hosts, develop high transmissibility in human populations, escape immunity, develop antiviral resistance, and achieve other phenotypic properties that have a bearing upon their disease-producing and epidemic/pandemic potential.

The DURC framework to guide decisions about the funding of H5N1 experiments is a significant step in addressing biosafety and biosecurity concerns (99). But while scientists and policy makers continue to grapple with safety issues, IAV continue to evolve in complex and unpredictable ways by breaking, as a matter of their adaptable ecobiology, all of the very same dual use rules. It is an irony that influenza viruses have survived to cause millions of cumulative deaths precisely because they have learned how to break our self-imposed safety "rules" and that these rules might someday have the unintended consequence of limiting the very research that can potentially decipher the most devastating of IAV's secrets and that allows us to develop critically needed preventive and therapeutic modalities.

The issue naturally arises as to whether, in our efforts to save lives and prevent disease, we are better off avoiding research designed to elucidate the mechanisms of IAV-induced disease and host switching or whether we should proceed in conducting this type of research in as safe a manner as is possible. Like so much else in science and public health, it comes down to weighing the risks and benefits. In this case, the risks of inaction are predictable: influenza pandemics, epidemics, and epizootics will continue to wreak havoc for the foreseeable future at the cost of countless lives. In considering the relative merits of supporting or thwarting safe research aimed at better preventing and controlling influenza by elucidating fundamental viral mechanisms, IAV's own actions should surely be borne in mind.

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