PERSPECTIVES: VIROLOGY

A Molecular Whodunit

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Two influenza outbreaks in the 20th century challenge current beliefs about patterns of influenza virulence. The "Spanish flu" pandemic of 1918, rather than sparing young healthy adults, killed millions in the prime of life. It wiped out entire villages at opposite ends of the Earth and depressed world population growth for 10 years. In 1997, a lethal avian influenza virus was transmitted directly to humans from chickens in Hong Kong. Six of 18 clinically diagnosed human cases were fatal, and, again, many of the victims were young adults. Both of these outbreaks suggest the emergence of highly virulent influenza variants. Unfortunately, until the basis of influenza virulence is understood, the human population will be defenseless against similar outbreaks in the future. In this issue of *Science*, Hatta and colleagues (page 1840) (*1*) and Gibbs and co-workers (page 1842) (*2*) offer new insights into the virulence of these influenza strains.

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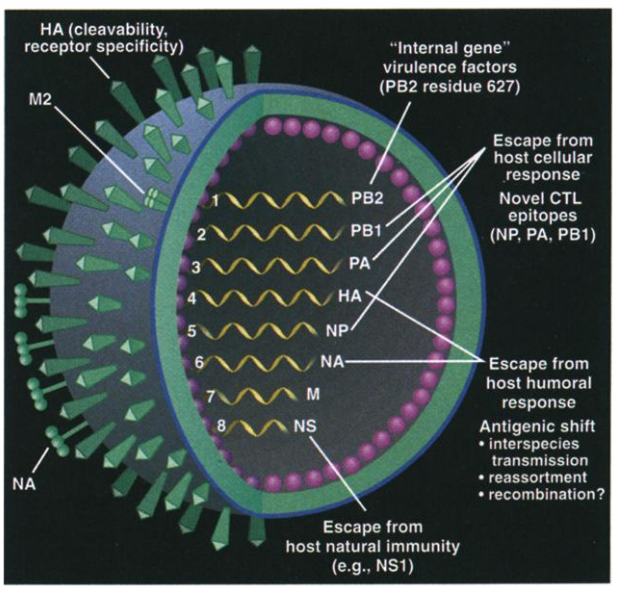
The virulence of a virus is defined by its comparative capacity to produce disease in a host (*3*). The 1918 Spanish flu virus was extremely virulent: It killed 10 times as many persons in the United States as did the 1957 Asian flu and about 20 times as many as the 1968 Hong Kong flu. Both the Asian and 1968 Hong Kong viruses were reassortants, that is, their genes were acquired from flu viruses infecting different host species. Genes encoding the hemagglutinin (HA) and polymerase 1 (PB1) proteins of these two flu viruses—and the enzyme neuraminidase (NA) of the Asian strain—were acquired from a Eurasian avian influenza virus; the remaining genes were all acquired from the human influenza virus circulating at the time.

The origin of the 1918 Spanish influenza virus, however, is still a work in progress. Taubenberger's group is analyzing short fragments of RNA from the tissues of 1918 victims: preserved specimens from soldiers and lung tissue from an Inuit woman buried in the Alaskan permafrost (*4*). Sequence and phylogenetic analysis of the HA, NA, and nonstructural (NS) gene segments of these samples suggests that an avian influenza virus was transmitted to humans and pigs, developing separate lineages sometime before 1918. The available data do not suggest that the 1918 virus is a reassortant, rather, it seems to be more akin to the "bird flu" that emerged in Hong Kong in 1997.

As many as 10% of poultry workers in Hong Kong were serologically reactive to the 1997 "bird flu" virus (subtype H5N1) (*5*). Late in that year, the deaths of 6 of 18 clinically diagnosed persons suggested that a variant that was highly virulent in humans had emerged. When the viruses isolated from humans were inoculated into mice, they differed in virulence: One group of viruses replicated in the lungs, spread to the brain, and was lethal, whereas the other replicated only in the lungs and did not cause death. Because there was a general correspondence between lethality in humans and in mice, the mouse offered an experimental system for dissection of the genetic basis of the virulence of these viruses.

Genetic manipulation of segmented negative-sense RNA viruses such as influenza virus was extremely difficult until 1989, when Palese and collaborators developed an appropriate reverse genetics method (*6*). Only in the past 2 years has it become possible to recover all eight gene segments of infectious influenza viruses from bacterial plasmids (*1*). Because these plasmid-only systems can be used in any laboratory, influenza viruses can now be "made to order." Taking advantage of plasmid-based reverse genetics, Hatta *et al.* (*1*) compared a pair of the mouse-lethal and mouse-nonlethal H5N1 influenza virus strains from Hong Kong. They show that a glutamic acid–to–lysine substitution at residue 627 of the PB2 polymerase protein, together with an HA glycoprotein that can be readily cleaved, determined the extreme virulence of the H5N1 Hong Kong flu virus. Interestingly, although mice are not men, the PB2 of all human influenza viruses (subtypes H1, H2, and H3) so far analyzed has a lysine at position 627, whereas only 3 of the 17 H5N1 human isolates from Hong Kong in 1997 had a lysine at this position. The H5N1 viruses isolated from humans were probably evolving rapidly because of their recent introduction to a new host.

The virulence of H5N1 in humans is likely to involve more genomic changes than the PB2 point mutation. Classical reassortant experiments indicate that the virulence of influenza virus is a polygenic trait involving HA and a constellation of other gene segments that can vary with the virus strain and host (see the figure) (*7*). Furthermore, although mice are a good model of human H5N1 infection (because the viruses replicate in mice without adaptation), the viruses are not transmitted from mouse to mouse. The wholesale slaughter of poultry in Hong Kong in 1997 eliminated the source of H5N1 from live poultry markets and interrupted any mutation and reassortment that would have permitted human-to-human spread. The Hatta *et al.* work is a big step forward because it defines the molecular basis of the virulence of two examples of the Hong Kong 1997 H5N1 virus in mice (*1*). But just as important is the fact that it provides a proof-of-principle that reverse genetics has finally come of age in influenza research.



The devil is in the details. The proteins of the influenza virus and their importance in virulence. The glycoprotein hemagglutinin (HA) is the principal antigen on the surface of the flu virus and is cleaved by proteases in host epithelial cells. The enzyme neuraminidase (NA), also on the flu virus surface, cleaves sialic acid residues from the host cell receptor for the virus, freeing virus particles and enabling them to spread throughout the body. A point mutation in the internal protein PB2 (a polymerase) is associated with the virulence of the 1997 Hong Kong flu virus (*1*). The NS1 protein is an interferon antagonist and blocks the host's ability to make interferon. For a virus to be highly virulent with pandemic potential, it must circumvent the host immune response. To do this, the virus must express new epitopes on its proteins (HA, NA, NP, PA, and PB1) that will not be recognized by the host's T and B cells.

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We do not yet know the basis of the virulence of the 1918 Spanish flu virus. In their report, Gibbs *et al.* (*2*) propose that a recombinant HA was responsible for the virulence of this virus. Their proposal is definitely a stretch for influenza virologists because homologous recombination (portions of a single gene segment from two different flu virus strains) is a rare event among RNA viruses, and many influenza virologists are not convinced that it even occurs. However, unorthodox proposals like this one can make everyone stop and reconsider the evidence. The authors contend that the Spanish influenza virus HA was a recombinant whose globular domain (HA1), which contains antigenic and host cell receptor binding sites, was acquired from a swine influenza virus and whose stalk region (HA2) was derived from the human virus. Their proposal that the 1918 swine lineage diverged from the human lineage before 1918 is consistent with the results of earlier phylogenetic analyses of the nucleoprotein (NP) and matrix (M) flu virus genes, which placed the divergence of the swine and human lineages several years before the pandemic (*8*, *9*). There is evidence that a much milder strain of the 1918 H1N1 virus was circulating before the pandemic began. Military medical records (long kept secret) reveal that there were a large number of deaths from respiratory infection in military camps in France in 1916 (*10*). The heliotrope cyanosis (bluish-purple discoloration of skin and mucous membranes) described in these records from 1916 is similar to that seen in 1918 flu victims. Therefore, the Spanish flu virus of 1918 or its precursor viruses are likely to have preceded the arrival of American troops in Europe, although the origin and route of the viruses are unknown. The widely distributed coincidental outbreaks of the Spanish flu in different parts of the world seem to correspond with the return of soldiers from Europe to their home countries at the end of World War I.

Definitive genetic analysis of the 1918 human influenza virus is difficult. The primary sequences of swine and avian influenza viruses before 1930 are unknown, and available samples of these viruses after 1930 have acquired mutations because of their passage in chicken eggs and mice. The transfer of viruses from pigs to humans or vice versa and the infection of either host with both pig and human viruses before 1918 would provide possible conditions for reassortment or recombination. Swine H1N1 virus is frequently transmitted to humans and occasionally causes human deaths (*11*). Influenza viruses are subject to different selective pressures in pigs and in humans—for example, HA undergoes antigenic drift (the accumulation of single amino acid changes) more slowly in pigs. Thus, although the highly pathogenic 1918 virus may have come from the pig lineage, the evidence is not conclusive. These questions may be resolved if archival samples containing swine influenza viruses can be found.

Unfortunately, the proposed recombination events through which the Spanish flu virus may have arisen bring us no closer to understanding its virulence. To be highly virulent, a virus must possess new B and T cell epitopes on its HA, NA, NP, PA, and PB1 proteins that have not been seen previously by the host lymphocyte population. In this way, the flu virus is able to rapidly invade host epithelial cells before the immune system has a chance to become mobilized. In addition, extreme virulence requires that the interaction of virus with host lymphocytes must trigger a devastating cytokine and apoptotic response resulting in severe inflammation and the death of large numbers of cells (*12*).

The parts played by PB2 and other proteins of the 1918 flu virus in the overwhelming immune responses that killed healthy young soldiers within a single day remain to be understood. The NS1 protein turns out to be a potent type I interferon antagonist (*13*). Whether NS1 was a crucial player in the virulence of the 1918 virus remains an open question. In preliminary studies, a highly laboratory-adapted reassortant strain of the A/WSN/33 (H1N1) virus containing the 1918 NS gene sequence was not virulent in mice (*4*).

These questions cannot be resolved until the entire primary sequence of the 1918 Spanish influenza genome is known. The sequencing and assembly of the shorter gene segments (HA, NA, and NS) from short RNA fragments is a major achievement. The sequencing and assembly of the larger PB1 and PB2 genes remains a huge challenge. Although it appears likely that the entire genome sequence will be obtained, the possibility of error will increase with the length of the genes, and multiple genomes must be analyzed to ensure an authentic sequence. Additional helpful clues might be obtained from the sequence of the causative agent of the mild influenza outbreak in early 1918 and from the genomes of other ancient avian or mammalian viruses that may be found in the permafrost. Efforts are under way to obtain frozen penguin and gull droppings from ancient nesting sites in the Antarctic permafrost.

Recent advances in reverse genetics, such as those described by Hatta *et al.*, now permit complete manipulation of all genes of the influenza virus. This progress offers many advantages. It is now possible to make human vaccines more quickly and efficiently by creating tailor-made rapid-growth, high-yield reassortants. Specific changes can be inserted into future live attenuated vaccine strains, and all functional domains of flu virus proteins, and their interactions with host cells, can be defined. Creation of the global influenza laboratory proposed by Layne and colleagues (see the editorial on page 1729) will provide advance warning of a new pandemic influenza virus that, together with reverse genetics and human genomics, will resolve the molecular basis of influenza virulence. That information in turn will allow the selection of vaccine strains with greater certainty and may in the future allow us to identify influenza viruses that are potential human pandemic strains.

Manipulation of influenza viruses by reverse genetics is also cause for caution. When the complete sequence of the 1918 virus is obtained, it may be possible to create the virus anew. Such a study should be attempted only if its benefits warrant the risk and if high-level biosafety laboratories are used. Of more immediate concern is the ability to make H5N1 "bird flu"–like viruses that can be transmitted among mammals. Although any influenza virus can theoretically arise in the natural environment, scientists will possess the knowledge and the tools to assemble viruses that are tailored for virulence in the desired host. Safety issues concerning the manipulation of influenza viruses by reverse genetics were explored at a National Institute of Allergy and Infectious Diseases (USA) conference in July this year. Discussions centered on using local biosafety committees to examine the specific planned work and to make risk assessments and safety recommendations. The need to reexamine the current biosafety guidelines in light of technical advances was also debated.

The human population is most vulnerable to influenza viruses that have new antigenic properties. It now takes about 6 months to prepare an appropriate vaccine. Although advances in reverse genetics will shorten this time, several months will still be needed to prepare a vaccine. During the period between detection of a pandemic strain and the availability of a vaccine, antiviral drugs will be essential (see the Perspective on page 1776). It is gravely disquieting that no action has yet been taken to create strategic stockpiles of such drugs.

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