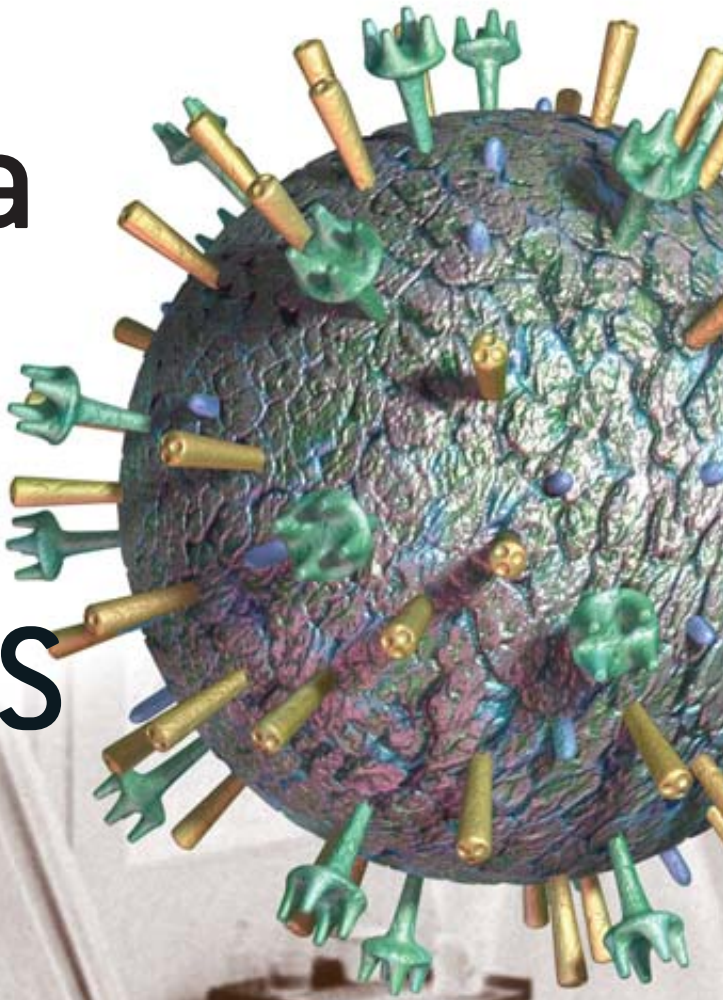
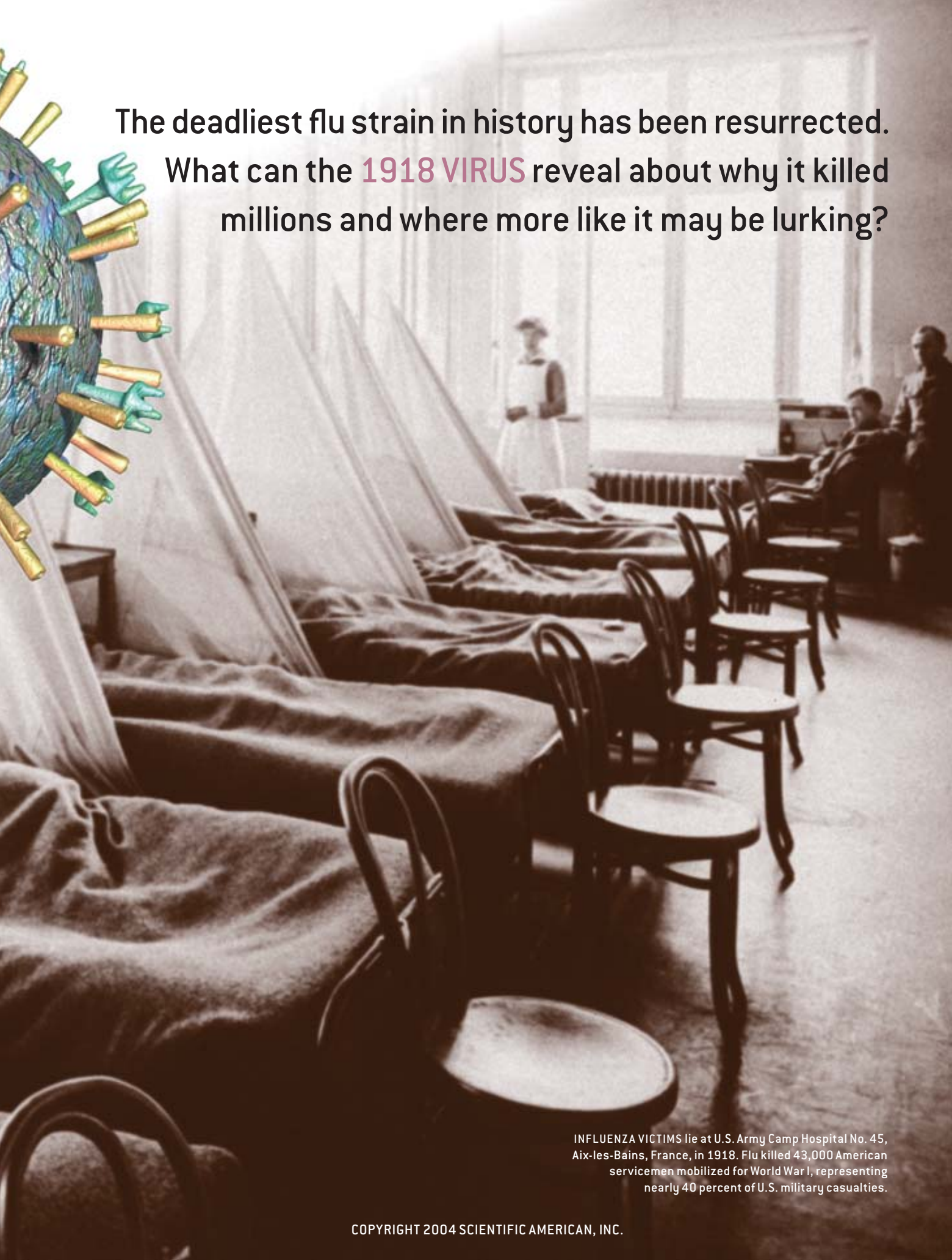
Capturing a Killer Flu VIRUS



By Jeffery K. Taubenberger, Ann H. Reid and Thomas G. Fanning



The deadliest flu strain in history has been resurrected. What can the 1918 VIRUS reveal about why it killed millions and where more like it may be lurking?

INFLUENZA VICTIMS lie at U.S. Army Camp Hospital No. 45, Aix-les-Bains, France, in 1918. Flu killed 43,000 American servicemen mobilized for World War I, representing nearly 40 percent of U.S. military casualties.

RED CROSS NURSES in St. Louis carry a flu patient in 1918. Health workers, police and a panicked public donned face masks for protection as the virus swept the country. Nearly a third of all Americans were infected during the pandemic, and 675,000 of them died.

On September 7, 1918, at the height of World War I, a soldier at an army training camp outside Boston came to sick call with a high fever. Doctors diagnosed him with meningitis but changed their minds the next day when a dozen more soldiers were hospitalized with respiratory symptoms. Thirty-six new cases of this unknown illness appeared on the 16th. Incredibly, by September 23rd, 12,604 cases had been reported in the camp of 45,000 soldiers. By the end of the outbreak, one third of the camp's population would come down with this severe disease, and nearly 800 of them would die. The soldiers who perished often developed a bluish skin color and struggled horribly before succumbing to death by suffocation. Many died less than 48 hours after their symptoms appeared, and at autopsy their lungs were filled with fluid or blood.

Because this unusual suite of symptoms did not fit any known malady, a distinguished pathologist of the era, William Henry Welch, speculated that "this must be some new kind of infection or plague." Yet the disease was neither plague nor even new. It was just influenza. Still, this particularly virulent and infectious strain of the flu virus is thought to have killed as many as 40 million people around the world between 1918 and 1919.

This most lethal flu outbreak in modern history disappeared almost as quickly as it emerged, and its cause was long believed lost to time. No one had preserved samples of the pathogen for later study because influenza would not be identified as a virus until the 1930s. But thanks to incredible foresight by the U.S. Army Medical Museum, the persistence of a pathologist named Johan Hultin, and advances in genetic analysis of old tissue samples, we have been able to retrieve parts of the 1918 virus and study their features. Now, more than 80 years after the horrible natural disaster of 1918–1919, tissues recovered from a handful of victims are answering fundamental questions both about the nature of this pandemic strain and about the workings of influenza viruses in general.

The effort is not motivated merely by historical curiosity. Because influenza viruses continually evolve, new influenza strains continually threaten human populations. Pandemic human flu viruses have emerged twice since 1918—in 1957 and 1968. And flu strains that usually infect only animals have also periodically caused disease in humans, as seen in the recent outbreak of avian influenza in Asia. Our two principal goals are determining what made the 1918 influenza so virulent, to guide development of influenza treatments and preventive measures, and establishing the origin of the pandemic virus, to better target possible sources of future pandemic strains.

Overview/*The Mystery of 1918*

- The flu pandemic that swept the globe in 1918–1919 was exceptional for the sheer numbers it killed, especially the number of young people who succumbed to the unusually virulent flu virus.
- What made the strain so deadly was a longstanding medical mystery until the authors devised techniques that allowed them to retrieve the 1918 virus's genes from victims' preserved tissues.
- Analysis of those genes and the proteins they encode revealed viral features that could have both suppressed immune defenses and provoked a violent immune reaction in victims, contributing to the high mortality.
- Known bird and mammal influenza hosts are unlikely sources of the pandemic virus, so its origin remains unsolved.

Hunting the 1918 Virus

IN MANY RESPECTS, the 1918 influenza pandemic was similar to others before it and since. Whenever a new flu strain emerges with features that have never been encountered by most people's immune systems, widespread flu outbreaks are likely. But certain unique characteristics of the 1918 pandemic have long remained enigmatic.

For instance, it was exceptional in both its breadth and depth. Outbreaks swept across Europe and North America, spreading as far as the Alaskan wilderness and the most remote islands of the Pacific. Ultimately, one third of the world's population may have been infected. The disease was also unusually severe, with death rates of 2.5 to 5 percent—up to 50 times the mortality seen in other influenza outbreaks.

GEORGE RETSECK (*flu virus*); CORBIS (*hospital*) (*preceding pages*); AMERICAN RED CROSS MUSEUM (*this page*)

By the fall of 1918 everyone in Europe was calling the disease the "Spanish" influenza, probably because neutral Spain did not impose the wartime censorship of news about the outbreak prevalent in combatant countries. The name stuck, although the first outbreaks, or spring wave, of the pandemic seemingly arose in and around military camps in the U.S. in March 1918. The second, main wave of the global pandemic occurred from September to November 1918, and in many places yet another severe wave of influenza hit in early 1919.

Antibiotics had yet to be discovered, and most of the people who died during the pandemic succumbed to pneumonia caused by opportunistic bacteria that infected those already weakened by the flu. But a subset of influenza victims died just days after the onset of their symptoms from a more severe viral pneumonia—caused by the flu itself—that left their lungs either massively hemorrhaged or filled with fluid. Furthermore, most deaths occurred among young adults between 15 and 35 years old, a group that rarely dies from influenza. Strikingly, people younger than 65 years accounted for more than 99 percent of all "excess" influenza deaths (those above normal annual averages) in 1918–1919.

Efforts to understand the cause of the 1918 pandemic and its unusual features began almost as soon as it was over, but the culprit virus itself remained hidden for nearly eight decades. In 1951 scientists from the University of Iowa, including a graduate student recently arrived from Sweden named Johan Hultin, went as far as the Seward Peninsula of Alaska seeking the 1918 strain [*see box on page 71*]. In November 1918 flu spread through an Inuit fishing village now called Brevig Mission in five days, killing 72 people—about 85 percent of the adult population. Their bodies had since been buried in permafrost, and the 1951 expedition members hoped to find the 1918 virus preserved in the victims' lungs. Unfortunately, all attempts to culture live influenza virus from these specimens were unsuccessful.

In 1995 our group initiated an attempt to find the 1918 virus using a different source of tissue: archival autopsy specimens stored at the Armed Forces Institute of Pathology (AFIP). For several years, we had been developing expertise in extracting fragile viral genetic material from damaged or decayed tissue for diagnostic purposes. In 1994, for instance, we were able to use our new techniques to help an AFIP marine mammal pathologist investigate a mass dolphin die-off that had been blamed on red tide. Although the available dolphin tissue samples were badly decayed, we extracted enough pieces of RNA from them to identify a new virus, similar to the one that causes canine distemper, which proved to be the real cause of the dolphin deaths. Soon we began to wonder if there were any older medical mysteries we might solve with our institute's resources.

A descendant of the U.S. Army Medical Museum founded in 1862, the AFIP has grown along with the medical specialty of pathology and now has a collection of three million specimens. When we realized that these included autopsy samples from 1918 flu victims, we decided to go after the pandemic virus. Our initial study examined 78 tissue samples from victims of the deadly fall wave of 1918, focusing on those with the severe lung damage characteristic of patients who died rapidly. Because the influenza virus normally clears the lungs just days after infection, we had the greatest chance of finding virus remnants in these victims.

The standard practice of the era was to preserve autopsy specimens in formaldehyde and then embed them in paraffin, so fishing out tiny genetic fragments of the virus from these 80-year-old "fixed" tissues pushed the very limits of the techniques we had developed. After an agonizing year of negative results, we found the first influenza-positive sample in 1996, a lung specimen from a soldier who died in September 1918 at Fort Jackson, S.C. We were able to determine the sequence of nucleotides in small fragments of five influenza genes from this sample.

But to confirm that the sequences belonged to the lethal 1918 virus, we kept looking for more positive cases and identified another one in 1997. This soldier also died in September 1918, at Camp Upton, N.Y. Having a second sample allowed us to confirm the gene sequences we had, but the tiny quantity of tissue remaining from these autopsies made us worry that we would never be able to generate a complete virus sequence.

A solution to our problem came from an unexpected source in 1997: Johan Hultin, by then a 73-year-old retired pathologist, had read about our initial results. He offered to return to Brevig Mission to try another exhumation of 1918 flu victims interred in permafrost. Forty-

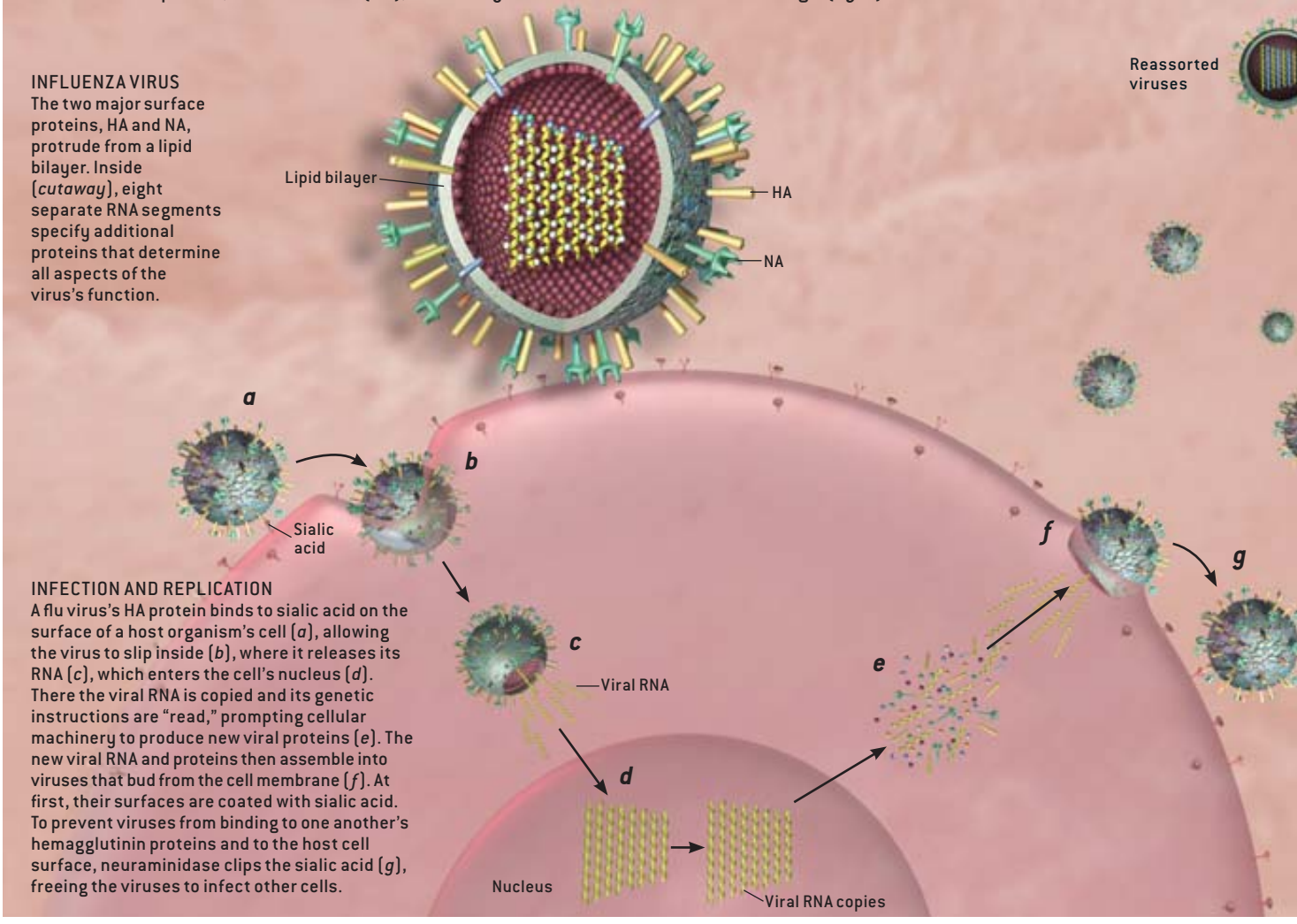
After an AGONIZING YEAR of negative results, we found THE FIRST CASE in 1996.

THE AUTHORS

JEFFERY K. TAUBENBERGER, ANN H. REID and *THOMAS G. FANNING* work together at the Armed Forces Institute of Pathology in Rockville, Md. In 1993 Taubenberger, a molecular pathologist, helped to create a laboratory there devoted to molecular diagnostics—identifying diseases by their genetic signatures rather than by the microscopic appearance of patients' tissue samples. Early work by Reid, a molecular biologist, led the group to devise the techniques for extracting DNA and RNA from damaged or decayed tissue that allowed them to retrieve bits and pieces of 1918 flu virus genes from archived autopsy specimens. Fanning, a geneticist with expertise in the evolution of genomes, helped to analyze the genes' relationships to other animal and human flu viruses. The authors wish to note that the opinions expressed in this article are their own and do not represent the views of the Department of Defense or the AFIP.

FLU HIJACKS HOSTS TO REPLICATE AND EVOLVE

Influenza is a small and simple virus—just a hollow lipid ball studded with a few proteins and bearing only eight gene segments (*below*). But that is all it needs to induce the cells of living hosts to make more viruses (*bottom*). One especially important protein on influenza's surface, hemagglutinin (HA), allows the virus to enter cells. Its shape determines which hosts a flu virus strain can infect. Another protein, neuraminidase (NA), cuts newly formed viruses loose from an infected cell, influencing how efficiently the virus can spread. Slight changes in these and other flu proteins can help the virus infect new kinds of hosts and evade immune attack. The alterations can arise through mistakes that occur while viral genes are being copied. Or they can be acquired in trade when the genes of two different flu viruses infecting the same cell intermingle (*right*).



INFLUENZA VIRUS
The two major surface proteins, HA and NA, protrude from a lipid bilayer. Inside (*cutaway*), eight separate RNA segments specify additional proteins that determine all aspects of the virus's function.

INFECTION AND REPLICATION
A flu virus's HA protein binds to sialic acid on the surface of a host organism's cell (*a*), allowing the virus to slip inside (*b*), where it releases its RNA (*c*), which enters the cell's nucleus (*d*). There the viral RNA is copied and its genetic instructions are "read," prompting cellular machinery to produce new viral proteins (*e*). The new viral RNA and proteins then assemble into viruses that bud from the cell membrane (*f*). At first, their surfaces are coated with sialic acid. To prevent viruses from binding to one another's hemagglutinin proteins and to the host cell surface, neuraminidase clips the sialic acid (*g*), freeing the viruses to infect other cells.

six years after his first attempt, with permission from the Brevig Mission Council, he obtained frozen lung biopsies of four flu victims. In one of these samples, from a woman of unknown age, we found influenza RNA that provided the key to sequencing the entire genome of the 1918 virus.

More recently, our group, in collaboration with British colleagues, has also been surveying autopsy tissue samples from 1918 influenza victims from the Royal London Hospital. We have been able to analyze flu virus genes from two of these cases and have found that they were nearly identical to the North American samples, confirming the rapid worldwide spread of a uniform virus. But what can the sequences tell us about the virulence and origin of the 1918 strain? Answering those questions requires a bit of background about how influenza viruses function and cause disease in different hosts.

Flu's Changing Face

EACH OF THE THREE novel influenza strains that caused pandemics in the past 100 years belonged to the type A group of flu viruses. Flu comes in three main forms, designated A, B and C. The latter two infect only humans and have never caused pandemics. Type A influenza viruses, on the other hand, have been found to infect a wide variety of animals, including poultry, swine, horses, humans and other mammals. Aquatic birds, such as ducks, serve as the natural "reservoir" for all the known subtypes of influenza A, meaning that the virus infects the bird's gut without causing symptoms. But these wild avian strains

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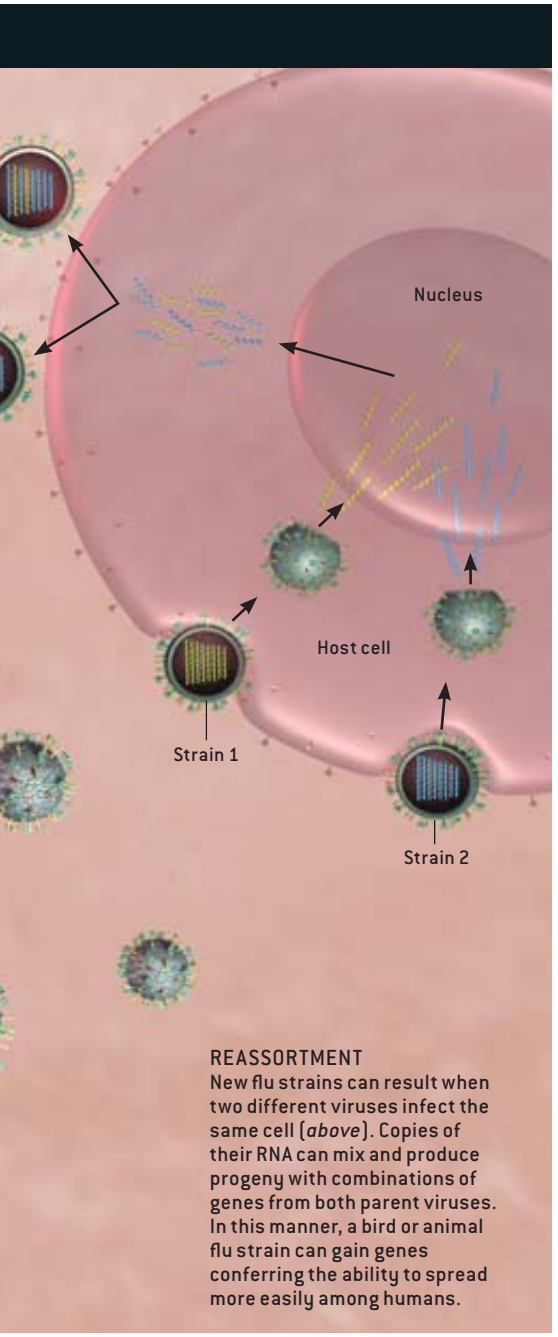


REASSORTMENT
New flu strains can result when two different viruses infect the same cell (*above*). Copies of their RNA can mix and produce progeny with combinations of genes from both parent viruses. In this manner, a bird or animal flu strain can gain genes conferring the ability to spread more easily among humans.

can mutate over time or exchange genetic material with other influenza strains, producing novel viruses that are able to spread among mammals and domestic poultry.

The life cycle and genomic structure of influenza A virus allow it to evolve and exchange genes easily. The virus's genetic material consists of eight separate RNA segments encased in a lipid membrane studded with proteins [*see top illustration on opposite page*]. To reproduce, the virus binds to and then enters a living cell, where it commandeers cellular machinery, inducing it to manufacture new viral proteins and additional copies of viral RNA. These pieces then assemble themselves into new viruses that escape the host cell, proceeding to infect other cells. No proofreading mechanism ensures that the RNA copies are accurate, so mistakes leading to new mutations are common. What is more, should two different influenza virus strains infect the same cell, their RNA segments can mix freely there, producing progeny viruses that contain a combination of genes from both the original viruses. This "reassortment" of viral genes is an important mechanism for generating diverse new strains.

Different circulating influenza A viruses are identified by referring to two signature proteins on their surfaces. One is hemagglutinin (HA), which has at least 15 known variants, or subtypes. Another is neuraminidase (NA), which has nine subtypes. Exposure to these proteins produces distinctive antibodies in a host, thus the 1918 strain was the first to be named, "H1N1," based on antibodies found in the bloodstream of pandemic survivors. Indeed, less virulent descendants of H1N1 were the predominant circulating flu strains until 1957, when an H2N2 virus emerged, causing a pandemic. Since 1968, the H3N2 subtype, which provoked the pandemic that year, has predominated.

The HA and NA protein subtypes present on a given influenza A virus are more than just identifiers; they are essential for viral reproduction and are primary targets of an infected host's immune system. The HA molecule initiates infection by binding to receptors on the surface of certain host cells. These tend to be respiratory lining cells in mammals and intestinal lining cells in birds. The NA protein enables new virus copies to escape the host cell so they can go on to infect other cells.

After a host's first exposure to an HA subtype, antibodies will block receptor binding in the future and are thus very effective at preventing reinfection with the same strain. Yet flu viruses with HA subtypes that are new to humans periodically appear, most likely through reassortment with the extensive pool of influenza viruses infecting wild birds. Normally, influenza HAs that are adapted to avian hosts bind poorly to the cell-surface receptors prevalent in the human respiratory tract, so an avian virus's HA binding affinity must be somewhat modified before the virus can replicate and spread efficiently in humans. Until recently, existing evidence suggested that a wholly avian influenza virus probably could not directly infect humans, but 18 people were infected with an avian H5N1 influenza virus in Hong Kong in 1997, and six died.

Outbreaks of an even more pathogenic version of that H5N1 strain became widespread in Asian poultry in 2003 and 2004, and more than 30 people infected with this virus have died in Vietnam and Thailand.

The virulence of an influenza virus once it infects a host is determined by a complex set of factors, including how readily the virus enters different tissues, how quickly it replicates, and the violence of the host's immune response to the intruder. Thus, understanding exactly what made the 1918 pandemic influenza strain so infectious and so virulent could yield great insight into what makes any influenza strain more or less of a threat.

A Killer's Face

WITH THE 1918 RNA we have retrieved, we have used the virus's own genes as recipes for manufacturing its component parts—essentially re-creating pieces of the killer virus itself. The first of these we were eager to examine was the hemagglutinin protein, to look for features that might explain the exceptional virulence of the 1918 strain.

We could see, for example, that the part of the 1918 HA that binds with a host cell is nearly identical to the binding site of a wholly avian influenza HA [*see illustration on page 69*]. In two of the 1918 isolates, this receptor-binding site differs from an avian form by only one amino acid building block. In the other three isolates, a second amino acid is also altered. These seemingly subtle mutations may represent the min-

REVERSE ENGINEERING THE FLU

When analyzing the genes of the 1918 virus revealed no definitive reasons for the pandemic strain's virulence, our group turned to reverse genetics—a method of understanding the function of genes by studying the proteins they encode. In collaboration with scientists from the Mount Sinai School of Medicine, the Centers for Disease Control and Prevention, the U.S. Department of Agriculture, the University of Washington and the Scripps Research Institute, we "built" influenza viruses containing one or more of the 1918 virus's genes, so we could see how these recombinant viruses behaved in animals and human cell cultures.

To construct these viruses, we employed a new technique called plasmid-based reverse genetics, which requires first making DNA copies of flu genes that normally exist in RNA form. Each DNA gene copy is then inserted into a tiny ring of DNA called a plasmid. Different combinations of these plasmids can be injected into living cells, where cellular machinery will execute the genetic instructions they bear and manufacture flu viruses with only the desired combination of genes.

Reverse genetics not only allows us to study the 1918 virus, it will allow scientists in the U.S. and Europe to explore how great a threat the H5N1 avian flu virus poses to humans. Since January 2004, that strain—which is now present in birds in 10 Asian countries—has infected more than 40 people, killing more than 30 of them. One of the casualties was a mother who is believed to have contracted the virus from her daughter, rather than directly from a bird.

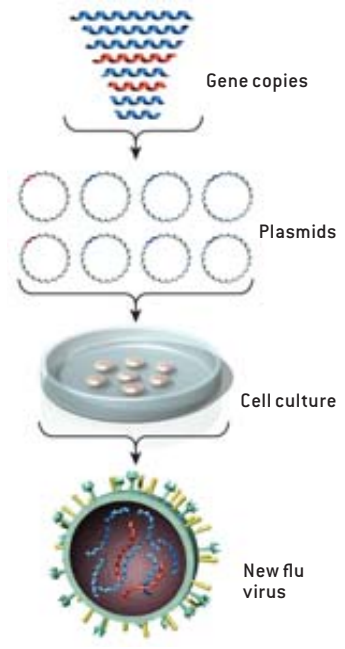
Such human-to-human transmission could suggest that in their case the avian virus had adapted to be more easily spread between humans, either by mutating or by acquiring new genes through reassortment with a circulating human flu strain. That dreaded development would increase the possibility of a human pandemic. Hoping to predict and thereby prevent such a disaster, scientists at the CDC and Erasmus University in the Netherlands are planning to test combinations of H5N1 with current human flu strains to assess the likelihood of their occurring naturally and their virulence in people.

What these experiments will reveal, as in our group's work with the 1918 virus genes, is crucial to understanding how influenza pandemics form and why they cause disease. Some observers have questioned the safety of experimenting with lethal flu strains, but all of this research is conducted in secure laboratories designed specifically to deal with highly pathogenic influenza viruses.

What is more, re-creating the 1918 virus proteins enabled us to establish that currently available antiviral drugs, such as amantadine or the newer neuraminidase inhibitors, such as oseltamivir (Tamiflu), would be effective against the 1918 strain in the case of an accidental infection. The H5N1 viruses are also sensitive to the neuraminidase inhibitors.

Scientists in the U.S. and U.K. also recently employed plasmid-based reverse genetics to create a seed strain for a human vaccine against H5N1. They made a version of the H5N1 virus lacking the wild strain's most deadly features, so that manufacturers could safely use it to produce a vaccine [see "The Scientific American 50," December 2004]. Clinical trials of that H5N1 vaccine were scheduled to begin at the end of 2004.

—J.K.T., A.H.R. and T.G.F.



PLASMID-BASED reverse genetics lets scientists custom-manufacture flu viruses. DNA copies of genes from two different flu strains (*blue* and *red*) are inserted into DNA rings called plasmids. The gene-bearing plasmids are then injected into a culture of living cells, which manufacture whole flu viruses containing the desired combination of genes.

imal change necessary to allow an avian-type HA to bind to mammalian-type receptors.

But while gaining a new binding affinity is a critical step that allows a virus to infect a new type of host, it does not necessarily explain why the 1918 strain was so lethal. We turned to the gene sequences themselves, looking for features that could be directly related to virulence, including two known mutations in other flu viruses. One involves the HA gene: to become active in a cell, the HA protein must be cleaved into two pieces by a gut-specific protein-cutting enzyme, or protease, supplied by the host. Some avian H5 and H7 subtype viruses acquire a gene mutation that adds one or more basic amino acids to the cleavage site, allowing HA to be activated by ubiquitous proteases. In chickens and other birds, infection by such a virus causes disease in multiple organs and even the central nervous system, with a very high mortality rate. This mutation has been observed in the H5N1 viruses currently circulating in Asia. We did not, however, find it in the 1918 virus.

The other mutation with a significant effect on virulence has been seen in the NA gene of two influenza virus strains that infect mice. Again, mutations at a single amino acid appear to allow the virus to replicate in many different body tissues, and these flu strains are typically lethal in laboratory mice. But we did not see this mutation in the NA of the 1918 virus either.

Because analysis of the 1918 virus's

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genes was not revealing any characteristics that would explain its extreme virulence, we initiated a collaborative effort with several other institutions to re-create parts of the 1918 virus itself so we could observe their effects in living tissues.

A new technique called plasmid-based reverse genetics allows us to copy 1918 viral genes and then combine them with the genes of an existing influenza strain, producing a hybrid virus. Thus, we can take an influenza strain adapted to mice, for example, and give it different combinations of 1918 viral genes. Then, by infecting a live animal or a human tissue culture with this engineered virus, we can see which components of the pandemic strain might have been key to its pathogenicity.

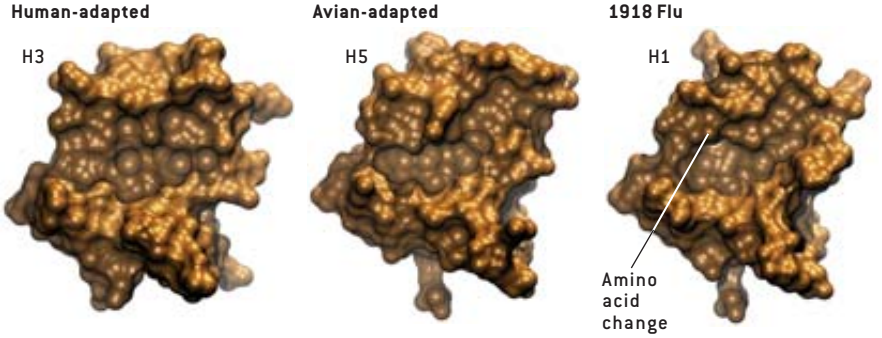
For instance, the 1918 virus's distinctive ability to produce rapid and extensive damage to both upper and lower respiratory tissues suggests that it replicated to high numbers and spread quickly from cell to cell. The viral protein NS1 is known to prevent production of type I interferon (IFN)—an "early warning" system that cells use to initiate an immune response against a viral infection. When we tested recombinant viruses in a tissue culture of human lung cells, we found that a virus with the 1918 NS1 gene was indeed more effective at blocking the host's type I IFN system.

To date, we have produced recombinant influenza viruses containing between one and five of the 1918 genes. Interestingly, we found that any of the recombinant viruses possessing both the 1918 HA and NA genes were lethal in mice, causing severe lung damage similar to that seen in some of the pandemic fatalities. When we analyzed these lung tissues, we found signatures of gene activation involved in common inflammatory responses. But we also found higher than normal activation of genes associated with the immune system's offensive soldiers, T cells and macrophages, as well as genes related to tissue injury, oxidative damage, and apoptosis, or cell suicide.

More recently, Yoshihiro Kawaoka of the University of Wisconsin–Madison reported similar experiments with 1918 flu genes in mice, with similar results. But when he tested the HA and NA genes separately, he found that only the 1918 HA produced the intensive immune response, suggesting that for reasons as yet unclear, this protein may have played a key role in the 1918 strain's virulence.

These ongoing experiments are providing a window to the past, helping scientists understand the unusual characteristics of the 1918 pandemic. Similarly, these techniques will be used to study what types of changes to the current H5N1 avian influenza strain might give that extremely lethal virus the potential to become pandemic in humans [*see box on opposite page*]. An equally compelling question is how such virulent strains emerge in the first place, so our group has also been analyzing the 1918 virus's genes for clues about where it might have originated.

Seemingly subtle mutations may allow an AVIAN hemagglutinin to bind to MAMMALIAN receptors.



HEMAGGLUTININ (HA) of the 1918 flu strain was re-created from its gene sequence by the authors' collaborators so they could examine the part that binds to a host cell's sialic acid and allows the virus to enter the cell. HA binding sites usually are shaped differently enough to bar cross-species infection. For instance, the human-adapted H3-type HA has a wide cavity in the middle of its binding site (*left*), whereas the avian H5 cavity (*center*) is narrow. The 1918 H1-type HA (*right*) more closely resembles the avian form, with only a few minor differences in the sequence of its amino acid building blocks. One of these alterations (*above right*) slightly widens the central cavity, apparently just enough to have allowed a flu virus with this avian-type HA to infect hundreds of millions of humans in 1918–1919.

Seeking the Source

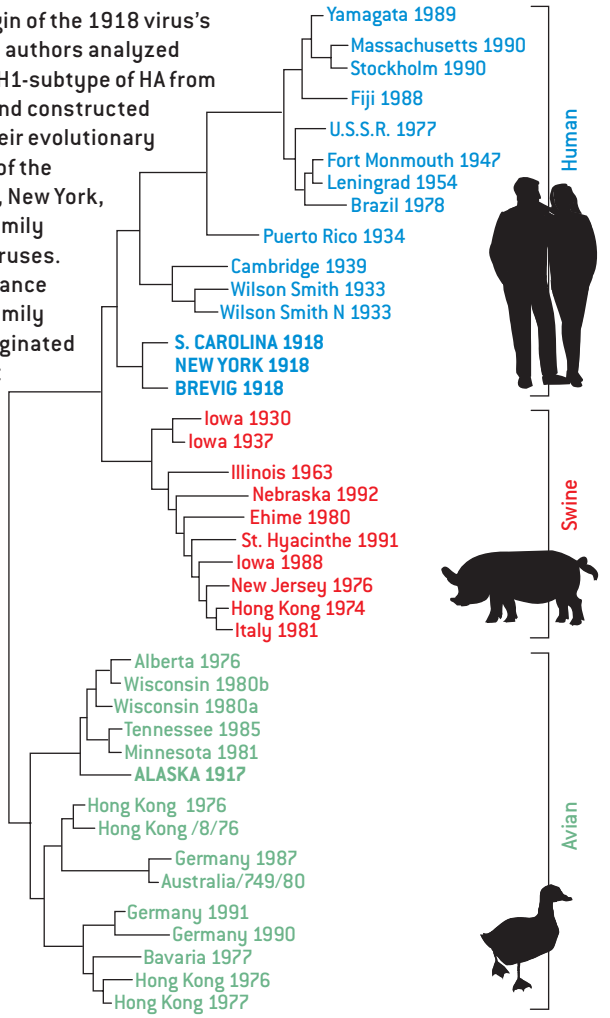
THE BEST APPROACH to analyzing the relationships among influenza viruses is phylogenetics, whereby hypothetical family trees are constructed using viral gene sequences and knowledge of how often genes typically mutate. Because the genome of an influenza virus consists of eight discrete RNA segments that can move independently by reassortment, these evolutionary studies must be performed separately for each gene segment.

We have completed analyses of five of the 1918 virus's eight RNA segments, and so far our comparisons of the 1918 flu genes with those of numerous human, swine and avian influenza viruses always place the 1918 virus within the human and swine families, outside the avian virus group [*see box on next page*]. The 1918 viral genes do have some avian features, however, so it is probable that the virus originally emerged from an avian reservoir sometime before 1918. Clearly by 1918, though, the virus had acquired enough adaptations to mammals to function as a human pandemic virus. The question is, where?

When we analyzed the 1918 hemagglutinin gene, we found that the sequence has many more differences from

Flu Family Tree

Seeking clues to the origin of the 1918 virus's hemagglutinin (HA), the authors analyzed gene sequences for the H1-subtype of HA from a variety of flu strains and constructed a phylogeny showing their evolutionary relationships. Samples of the 1918 strain (S. Carolina, New York, Brevig) fell within the family of human-adapted flu viruses. The 1918 H1 gene's distance from the known avian family could indicate that it originated in an avian flu strain but spent time evolving in an unidentified host before emerging in 1918. Supporting this conclusion, a contemporary avian strain found in a preserved Brant goose (Alaska 1917) was evolutionarily distant from the 1918 strain and more similar to modern bird flus.



avian sequences than do the 1957 H2 and 1968 H3 subtypes. Thus, we concluded, either the 1918 HA gene spent some length of time in an intermediate host where it accumulated many changes from the original avian sequence, or the gene came directly from an avian virus, but one that was markedly different from known avian H1 sequences.

To investigate the latter possibility that avian H1 genes might have changed substantially in the eight decades since the 1918 pandemic, we collaborated with scientists from the Smithsonian Institution's Museum of Natural History and Ohio State University. After examining many preserved birds from the era, our group isolated an avian subtype H1 influenza strain from a Brant goose collected in 1917 and stored in ethanol in the Smithsonian's bird collections. As it turned out, the 1917 avian H1 sequence was closely related to modern avian North American H1 strains, suggesting that avian H1 sequences have changed little over the past 80 years. Extensive sequencing of additional wild bird H1 strains may yet identify a strain more similar to the 1918 HA, but it may be that no avian H1 will be found resembling the 1918 strain because, in fact, the HA did not reassort directly from a bird strain.

In that case, it must have had some intermediate host. Pigs are a widely suggested possibility because they are known to be susceptible to both human and avian viruses. Indeed, simultaneous outbreaks of influenza were seen in humans and swine during the 1918 pandemic, but we believe that the direction of transmission was most probably from humans to pigs. There are numerous examples of human influenza A virus strains infecting swine since 1918, but swine influenza strains have been isolated only sporadically from humans. Nevertheless, to explore the possibility that the 1918 HA may have started as an avian form that gradually adapted to mammalian hosts in swine, we looked at a current example of how avian viruses evolve in pigs—an avian H1N1 influenza lineage that has become established in European swine over the past 25 years. We found that even 20 years of evolution in swine has not resulted in the number of changes from avian sequences exhibited by the 1918 pandemic strain.

When we applied these types of analyses to four other 1918 virus genes, we came to the same conclusion: the virus that sparked the 1918 pandemic could well have been an avian strain that was evolutionarily isolated from the typical wild waterfowl influenza gene pool for some time—one that, like the SARS coronavirus, emerged into circulation among humans from an as yet unknown animal host.

Future Investigations

OUR ANALYSES of five RNA segments from the 1918 virus have shed some light on its origin and strongly suggest that the pandemic virus was the common ancestor of both subsequent human and swine H1N1 lineages, rather than having emerged from swine. To date, analyzing the viral genes has offered no definitive clue to the exceptional virulence of the 1918 virus strain. But experiments with engineered viruses containing 1918 genes indicate that certain of the 1918 viral proteins could promote rapid virus replication and provoke an intensely destructive host immune response.

In future work, we hope that the 1918 pandemic virus strain can be placed in the context of influenza viruses that immediately preceded and followed it. The direct precursor of the pandemic virus, the first or spring wave virus strain, lacked the autumn wave's

JOHNNY JOHNSON

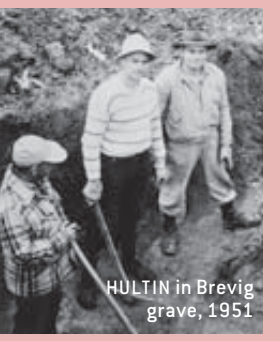
exceptional virulence and seemed to spread less easily. At present, we are seeking influenza RNA samples from victims of the spring wave to identify any genetic differences between the two strains that might help elucidate why the autumn wave was more severe. Similarly, finding pre-1918 human influenza RNA samples would clarify which gene segments in the 1918 virus were completely novel to humans. The unusual mortality among young people during the 1918 pandemic might be explained if the virus shared features with earlier circulating strains to which older people had some immunity. And finding samples of H1N1 from the 1920s and later would help us understand the 1918 virus's subsequent evolution into less virulent forms.

We must remember that the mechanisms by which pandemic flu strains originate are not yet fully understood. Because the 1957 and 1968 pandemic strains had avian-like HA proteins, it seems most likely that they originated in the direct reassortment of avian and human virus strains. The actual circumstances of those reassortment events have never been identified, however, so no one knows how long it took for the novel strains to develop into human pandemics.

The 1918 pandemic strain is even more puzzling, because its gene sequences are consistent neither with direct reassortment from a known avian strain nor with adaptation of an avian strain in swine. If the 1918 virus should prove to have acquired novel genes through a different mechanism than subsequent pandemic strains, this could have important public health implications. An alternative origin might even have contributed to the 1918 strain's exceptional virulence. Sequencing of many more avian influenza viruses and research into alternative intermediate hosts other than swine, such as poultry, wild birds or horses, may provide more clues to the 1918 pandemic's source. Until the origins of such strains are better understood, detection and prevention efforts may overlook the beginning of the next pandemic. SA

Persistence Pays Off

Visiting Alaska in the summer of 1949, Swedish medical student Johan Hultin met Lutheran missionaries in Fairbanks who told him of the 1918 flu pandemic's toll on Inuit villages. One, a tiny settlement on the Seward Peninsula called Teller Mission, was all but wiped out in November 1918. Overwhelmed missionaries had to call in the U.S. Army to help bury 72 victims' bodies in a mass grave, which they marked by two crosses.


HULTIN in Brevig grave, 1951

Haunted by the story, Hultin (*right, center* and *below*) headed to the University of Iowa to begin his doctoral studies in microbiology. There he kept thinking about the 1918 pandemic and wondering if the deadly virus that caused it could be retrieved for study from bodies that may have been preserved by the Alaskan permafrost. In the summer of 1951, Hultin convinced two Iowa faculty, a virologist and a pathologist, to visit the village, then called Brevig Mission. With permission from tribal elders, the scientists excavated the grave and obtained tissue specimens from what remained of several victims' lungs.

Back in Iowa, the team tried and tried to grow live virus from the specimens but never could. In retrospect, that was perhaps just as well since biological containment equipment for dangerous pathogens did not exist at the time.

Hultin's disappointment led him to abandon his Ph.D. and become a pathologist instead. Retired and living in San Francisco in 1997, Hultin read our group's first published description of the 1918 genes we retrieved from autopsy specimens, and it rekindled his hope of finding the entire 1918 virus. He wrote to me, eager to try to procure new lung specimens from Brevig Mission for us to work with. He offered to leave immediately for Alaska, and I agreed.

At the same time, Hultin tracked down his 1951 expedition mates to ask if they had kept any of the original Brevig specimens. We reasoned that those tissue samples obtained just 33 years after the pandemic and then preserved might be in better condition than specimens taken later. As it turned out, one of Hultin's colleagues had kept the material in storage for years but finally deemed it useless and threw it out. He had disposed of the last specimens just the year before, in 1996.

Fortunately, Hultin once again got permission from the Brevig Mission Council to excavate the 1918 grave in August 1997. And this time he found the body of a young woman who had been obese in life. Hultin said later that he knew instantly her tissue samples would contain the 1918 virus—together with the cold temperature, her thick layer of fat had almost perfectly preserved her lungs. He was right, and her tissue provided us with the entire genome of the 1918 pandemic virus. —*J.K.T.*


HULTIN in Brevig grave, 1997

MORE TO EXPLORE

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ASSOCIATED PRODUCERS (*top*), JOHAN HULTIN (*bottom*)