A Molecular Whodunit
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Published by: American Association for the Advancement of Science
Stable URL: http://www.jstor.org/stable/3084662
Accessed: 11-09-2015 19:24 UTC

REFERENCES
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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.

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) (3). Late in that year, the deaths of 6 of 18 clinically diagnosed persons suggested that a variant 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 may 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, where-
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 recombiant HA was responsible for the viru-

lence of this virus. Their proposal is defini-
tively a stretch for influenza virologists be-
cause 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 influen-
za virus HA was a recombinant whose globu-
lar domain (HA1), which contains antigenic

and host cell receptor binding sites, was ac-
quired 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 lin-
eage before 1918 is consistent with the re-

sults 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 be-

fore the pandemic (8, 9). There is evidence

that a much milder strain of the 1918 H1N1

flu virus was circulating before the pandemic be-
gan. Military medical records (long kept se-
cret) 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 like-

ly 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 sol-
diers from Europe to their home countries at

the end of World War I.

Definitive genetic analysis of the 1918 hu-

man influenza virus is difficult. The primary

sequences of swine and avian influenza virus-

es before 1930 are unknown, and available

samples of these viruses after 1930 have ac-
quired mutations because of their passage in

chicken eggs and mice. The transfer of virus-
es from pigs to humans or vice versa and the

infection of either host with both pig and hu-

man viruses before 1918 would provide pos-
ible conditions for reassortment or recombi-
nation. Swine H1N1 virus is frequently trans-
mitted to humans and occasionally causes hu-

man deaths (11). Influenza viruses are subject
to different selective pressures in pigs and in

humans—for example, HA undergoes anti-
genic drift (the accumulation of single amino

acid changes) more slowly in pigs. Thus, al-

though the highly pathogenic 1918 virus may

have come from the pig lineage, the evidence

is not conclusive. These questions may be re-

solved if archival samples containing swine

influenza viruses can be found.

Unfortunately, the proposed recombina-
tion events through which the Spanish flu

virus may have arisen bring us no closer to

understanding its virulence. To be highly viru-

lent, 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 ep-

ithelial cells before the immune system has a

chance to become mobilized. In addition, ex-

treme virulence requires that the interaction of

viruses 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 pro-
teins of the 1918 flu virus in the over-

whelming 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 cru-

cial player in the virulence of the 1918

flu virus remains an open question. In prelimi-

nary 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 Spa-

nish influenza genome is known. The sequenc-
ing and assembly of the shorter gene seg-

ments (HA, NA, and NS) from short RNA

fragments is a major achievement. The se-

quencing and assembly of the larger PB1 and

PB2 genes remains a huge challenge. Al-

though 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 ana-
lized to ensure an authentic sequence. Ad-

ditional 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 ob-

tain frozen penguin and gull droppings from an-

cient 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 in-

fluenza virus. This progress offers many ad-

vantages. It is now possible to make human

vaccines more quickly and efficiently by cre-

ating tailor-made rapid-growth, high-yield re-

assortants. 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 lab-

oratory proposed by Layne and colleagues

(see the editorial on page 1729) will provide

advance warning of a new pandemic influen-

tza virus that, together with reverse genetics

and antiviral drugs, will inform the molecu-

lar basis of influenza virulence. That infor-

mation in turn will allow the selection of vac-

cine strains with greater certainty and may in

the future allow us to identify influenza virus-

es that are potential human pandemic strains.

Manipulation of influenza viruses by re-

verse 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 im-

mediate 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 en-

vironment, scientists will possess the knowl-

dedge 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 ex-

plored 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 re-

examine the current biosafety guidelines in

light of technical advances was also debated.

The human population is most vulnera-

table to influenza viruses that have new anti-

genetic 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, an-
tiviral drugs will be essential (see the Per-

spective on page 1776). It is gravely dis-

quieting that no action has yet been taken to

create strategic stockpiles of such drugs.

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