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Question and Answer

Q&A: What do we know about influenza and what can we do about it?

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Influenza pandemics occur when human populations are infected by a variant virus to which a population has no prior immunity. What are the crucial varying genes?

The crucial genes are those encoding the two viral surface proteins hemagglutinin (H or HA) and neuraminidase (N or NA). The influenza A viruses [1] that infect mammals like us replicate principally in the epithelial cells of the airways. The HA facilitates viral entry by binding to sialic acid residues on the epithelial cell surface, while the NA functions to cleave such attachments, and so release new virus particles, or virions, both from the cell and from the slimy mucous that protects the lung and trachea. The new virions are then free to spread the infection, both from cell to cell and to other susceptible individuals. Antibodies that bind to either the HA or the NA and block their function effectively prevent (or terminate) the infectious process and thus provide protective immunity. The anti-influenza drugs zanamivir and oselatamivir (Relenza and Tamiflu) operate by blocking the NA active site and, as this was first characterized by the structural analysis of NA-antibody complexes, are among the earliest examples of rational drug design.

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The variability of the HA and NA proteins is due to lack of proof reading by the viral polymerase that leads in turn to poor fidelity of genome copying and frequent occurrence of mutations. The resulting virus variants are then subjected to selective pressure by neutralizing (blocking) antibodies produced by immune individuals, leading to the emergence of so-called escape mutants that are not detected by antibodies against the original virus and cause annual, or biennial, 'seasonal' influenza outbreaks. Such a virus can spread across the USA within a single month.

New HA and NA types also enter the human population as a consequence of genetic reassortment between, for example, viruses that have been circulating in humans and those that circulate in birds. The influenza virus genome is organized in eight discrete segments and, if a single cell is infected simultaneously with a 'human' and an 'avian' virus, the segments can become re-packaged to give a novel variant that could, for instance, express completely new (to humans) avian HA or NA types but whose other genes remain adapted to enable them to spread in people. Aquatic birds, which are the main reservoir of the influenza A viruses, are known to carry 16 different HAs and 9 NAs. Pigs, which can be infected with both avian and human viruses, are thought commonly to be the host from which reassortant influenza viruses emerge (Figure 1).

The three viruses that circulated in people through the 20th century were H1N1, H2N2 and H3N2. These crossed over into humans some time before 1918 (H1N1), in 1957 (H2N2) and in 1968 (H3N2), with all three causing pandemics. By far the worst was the 1918 H1N1 virus that killed some 40-70 million in a global population that was less than a third the size of that today. Though the first human influenza A virus was not isolated until 1933, the 1918 virus has been reconstructed by PCR from preserved lung tissues and from exhuming people who were buried in the Alaskan permafrost. Extremely virulent in rodents, ferrets and non-human primates, it has the characteristics of a mutant bird virus. Both the H2N2 "Asian 'Flu" and the 1968 "Hong Kong 'flu" are though to have originated by reassortment between mutant duck viruses and human viruses, with swine being the adapting host.

There is ample evidence that the H1N1 and H3N2 viruses have gone back and forth between humans and pigs, with the current 'swine' H1N1 being, perhaps, a descendant of the 1918 'human' virus. Some 12 cases of human infection with H1N1 viruses that have 'human', 'swine' and 'avian' genetic elements have been recognized in the USA since 1998, with 5,123 US cases identified to May 19 in the present outbreak (the world total of confirmed cases in 40 countries to that date being 9,830) [2]. Other viruses (for example, H9N2, H7N7 and H5N1) jump occasionally and cause severe

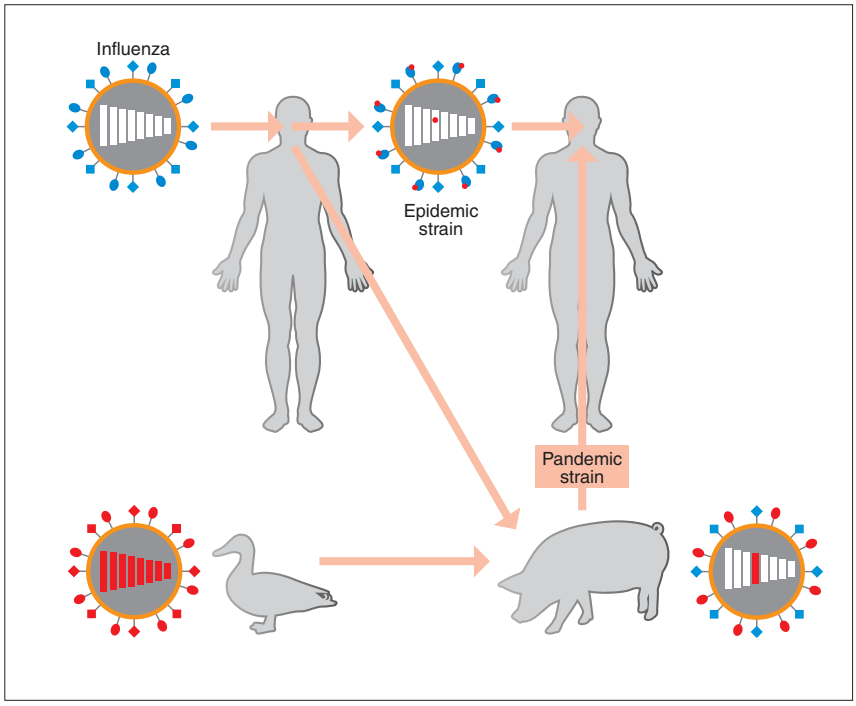

Figure 1
Antigenic drift and antigenic shift in different hosts of influenza virus. The surface hemagglutinin and neuraminidase molecules (blue) of influenza viruses undergo frequent mutation (antigenic drift) in their human hosts, giving rise to new variants (red dots) that can elude antibodies made in many individuals against the parent virus. Less frequently, entire segments of the eight-segment genome of an avian influenza virus and a human virus become reassorted into the same virion, usually through infection of swine by both viruses, and this can result in a virus that is still adapted to infect humans but expresses an avian hemagglutinin or neuraminidase (antigenic shift) to which there is no prior immunity in human populations. Figure reproduced with permission from Figure 10-17 of: DeFranco AD, *et al. Immunity* Oxford University Press; 2007.

disease in humans infected directly from birds, but have not to date been transmitted between humans.

What determines whether new animal virus variants that can pass from animals to humans can also be transmitted from human to human?

The primary factor is the sialic acid on the galactose on the surface of respiratory tract epithelial cells. HAs of human or pig viruses preferentially recognize sialic acid bound to galactose via an α2,6 linkage, while the bird virus HAs recognize an α2,3 linkage. Humans express only the α2,6 form in the upper respiratory tract, and the α2,3 forms only deep in the human lung (along with the α2,6 forms) [3]. Thus, breathing in a relatively light dose of an avian virus such as the H5N1 virus is unlikely to lead to infection, and it is thought that the occasional, often lethal (>60%) case of human H5N1 virus pneumonia results from very close exposure to an infected bird, allowing virus penetration to the bronchi and bronchioles.

The characteristics of the sialic acid linkage do not, however, seem to be the sole determinant limiting interspecies spread. Transmission experiments with ferrets, which have a receptor distribution comparable to that found in humans, suggest that changes in other genes may also be critical for determining infectivity. There is emerging evidence that elements of the three-component viral polymerase complex (PB1 and PB2) can influence transmissibility [4], though the underlying mechanism of host specificity in this case is not clear.

Can the ability of a viral variant to pass from human to human be predicted from its nucleic acid sequence?

This may be possible in the future and progress is being made in identifying conserved amino acid sequences associated with past pandemics [5] but we don't yet know enough about what determines infectivity and virulence to predict the key correlates of transmissibility just from the viral RNA sequence.

What determines the severity of disease caused by a given influenza virus?

The severity of influenza reflects both the characteristics of the infecting virus and host factors. Important host factors include age, basic health status and prior exposure to the same or related viruses, with the very young, the elderly, pregnant women and those who are otherwise clinically compromised being particularly susceptible in non-pandemic, seasonal influenza outbreaks. Secondary bacterial infection can also play a major part and there is a good case for ensuring that groups at greatest risk are given both influenza and pneumococcal vaccines. The nature of the early immune response to the virus is widely believed to be a major factor: paradoxically, the more vigorous it is, the greater the risk of mortality. Neutralizing antibodies, which are purely protective, take several days to produce. But so-called innate immune defenses are activated within minutes to hours of infection [6], and involve the production of inflam-

matory cytokines that cause increased vascular permeability and edema, as well as an influx of immune cells causing tissue destruction, with disastrous consequences for lung function. Such a so-called cytokine storm effect was first recognized in people infected with an avian H5N1 virus, and is likely to have been at least part of the reason for the excessive death rates in otherwise healthy young adults during the 1918 pandemic.

Other factors affecting virulence are the production of viral proteins capable of inhibiting host antiviral mechanisms - for example, the NS1 protein produced by the influenza virus inhibits the production of type I interferon, which is normally induced by viral infection and in turn induces cellular anti-viral proteins that interfere with viral replication; and we have already mentioned the HA and the NA, and members of the viral polymerase complex. Apart from their effects on viral infectivity and replicative capacity, the way that these genes operate to cause more severe disease is poorly understood.

Can the pathogenicity of an influenza virus be determined from its genome sequence?

Not yet, maybe some day.

Why does it take so long to make a vaccine against a new influenza virus variant?

The first decision that has to be made is: which vaccine? Vaccines that are used against seasonal influenza are based on the three most prevalent circulating influenza viruses. A comprehensive, international 'virus-watch' program based administratively in WHO Geneva co-ordinates the operations of four WHO Collaborating Centers (London, Melbourne, Tokyo, Atlanta) and a host of National Laboratories. A combination of RT-PCR and rapid sequencing is used for the rapid characterization of viruses in human circulation and that information is made available globally. A key WHO committee meets twice each year to decide which influenza A and B viruses will be included in the three-component (H1N1, H3N2, influenza B) vaccines manufactured commercially for use in the Northern and Southern hemispheres. Of course, this dynamic changes immediately when a new virus, like the current 'swine' H1N1, suddenly enters the human population raising the possibility that a new vaccine must be produced.

The most efficient, in terms of the amount of product required, are the so-called live-attenuated vaccines that have been adapted to replicate poorly so that they do not cause disease. Live-attenuated influenza virus vaccines were long in use in the former Russian Federation, but their broader availability is comparatively recent. Any vaccine that is capable of some replication has the advantage that it can be used at lower titer, but the disadvantage that its safety is less secure than that of a killed one. For this reason, such vaccines are not currently recommended for the very young or the elderly. There is also the difficulty that, if there is any cross-reactive neutralizing antibody, the vaccine dose will be too low to boost immunity. Attempts at making recombinant protein vaccines for influenza have so far been unsuccessful, principally because the proteins do not fold appropriately.

The more commonly used killed vaccines are made from viruses inactivated in formalin or β-propiolactone and used either as a whole virus or as a so-called split virus, in which the viral components are disrupted, or from which the HA and NA subunits are purified. High titer stocks are required as starting material for such vaccines. Although efforts are being made to develop cell-culture systems for producing large amounts of influenza virus, the optimal 'culture flask' is still the hen's egg. This requires large numbers of eggs and a specialized production facility, neither rapidly scalable, with such operations currently being used for about 6 months a year to produce sequentially the three batches of different viruses that go into the standard trivalent vaccine. If the new 'swine' H1N1 virus continues to spread and evolve, and is as different as it seems to be from the long-circulating 'human' H1N1 viruses, then we may need to think in terms of incorporating a fourth component, adding to the time required.

The vaccine reserve that was made to combat the possible H5N1 threat took even longer, because the H5N1 viruses were so virulent that they killed chick embryos before much virus was made, and new recombinant vaccine viruses had to be made by inserting the H5 and N1 into one of the standard vaccine strains. Even though it was inactivated, this 'genetically modified organism' had to go through the full range of phase 1 to 3 trials before it could be approved for human use.

As it is, the world has, with a current population of about 6.8 billion, never made more than about 400 million doses of trivalent influenza vaccine. For this reason, a great deal of current research is focused on identifying improved adjuvants - substances that increase the strength of the immune response, so that the vaccine is more effective and smaller amounts of viral protein are required [7].

Would it be possible in principle to make a vaccine that would protect against any new influenza virus variant?

The holy grail with influenza immunization, and for those trying to make vaccines against HIV and hepatitis C

virus, is to identify a component of the virus that is both accessible to antibody and cannot be changed because it plays some key functional role for the virus. It is possible to make monoclonal antibodies in the laboratory (mAbs) that prevent infection by binding to a highly conserved pocket in the HA stem region [8]. Analogous mAbs have been found for HIV. What we don't yet know, however, is how to make a vaccine that induces the human immune system to make these antibodies. Even so, mAbs produced artificially might be used for therapy or prophylaxis in the face of a novel, rapidly spreading, severe influenza pandemic. In the absence of a vaccine, it would be much more realistic to give, say, front-line medical personnel a monthly dose of a protective, humanized mAb rather than daily treatment with anti-viral drugs. The advantage of such an approach is that the mAbs could be stockpiled ahead of time, instead of having to be made anew each year, because the target site on the HA does not change.

Is there any other way to make a broadly protective vaccine?

When it comes to cross-reactive immunity, a possible target is the conserved, low abundance M2e protein (a proton selective channel) on the surface of the virion. Immunization with M2e has some protective efficacy in mice [9], but it is not clear whether this approach will work in humans. Another possibility is to develop a vaccine that instead of inducing antibodies activates the production of cytotoxic T lymphocytes. These effector T cells recognize and destroy virus-infected cells, which betray the infecting virus by displaying peptides derived from viral components bound to self-major histocompatibility complex molecules that are expressed on the surface of all cells. Because these peptides are often derived from conserved internal components of the virus, a vaccine based on them should be effective against many viral variants. This strategy has been shown to provide some cross-protection against HA- and NA-different viruses in mice [10]. Such immunity is not immediate, however, because the T cells must be reactivated from a resting/memory to an effector/cytotoxic state on re-exposure to the virus. The net consequence in mice is more rapid virus clearance and less severe disease. Many practical and regulatory issues arise, however, in connection with such a possible partially protective vaccine. Perhaps the T cell and M2e approaches might be combined in one product to provide a strategic reserve that could be made in large amounts ahead of a possible pandemic.

Where can I find out more?

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