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Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus

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The Spanish influenza pandemic of 1918–1919 caused acute illness in 25–30% of the world's population and resulted in the death of 40 million people. The complete genomic sequence of the 1918 influenza virus will be deduced using fixed and frozen tissues of 1918 influenza victims. Sequence and phylogenetic analyses of the complete 1918 haemagglutinin (HA) and neuraminidase (NA) genes show them to be the most avian-like of mammalian sequences and support the hypothesis that the pandemic virus contained surface protein-encoding genes derived from an avian influenza strain and that the 1918 virus is very similar to the common ancestor of human and classical swine H1N1 influenza strains. Neither the 1918 HA genes nor the NA genes possessed mutations that are known to increase tissue tropicity, which accounts for the virulence of other influenza strains such as A/WSN/33 or fowl plague viruses. The complete sequence of the non-structural (NS) gene segment of the 1918 virus was deduced and tested for the hypothesis that the enhanced virulence in 1918 could have been due to type I interferon inhibition by the NS1 protein. The results from these experiments were inconclusive. Sequence analysis of the 1918 pandemic influenza virus is allowing us to test hypotheses as to the origin and virulence of this strain. This information should help to elucidate how pandemic influenza strains emerge and what genetic features contribute to their virulence.

Keywords: influenza; pandemic; virulence; haemagglutinin; neuraminidase

'Causa latet, vis est notissima
(The cause is hidden, but the effect is obvious)'
Ovid, *Metamorphoses*, Book IV

1. INTRODUCTION

Influenza A viruses, as negative-strand RNA viruses of the Orthomyxoviridae, continually circulate in humans in yearly epidemics (mainly in the winter in temperate climates) and antigenically novel strains emerge sporadically as pandemic viruses (Cox & Subbarao 2000). Influenza kills 20 000 people in an average year in the USA (Simonsen *et al*. 2000). Influenza epidemics boost the yearly number of deaths past the average every 2 or 3 years, causing 10 000–15 000 additional deaths. Influenza occasionally and unpredictably sweeps the world, infecting 20–40% of the population in a single year. In these pandemic years, that have occurred every 10–40 years for at least several centuries, the numbers of deaths can be dramatically above average. In 1957–1958, a pandemic caused 66 000 excess deaths in the USA (Simonsen *et al*. 1998). The worst pandemic in recorded history in 1918 caused a total of *ca*. 675 000 deaths in the USA (United States Department of Commerce 1976) and killed 20–40 million people worldwide (Crosby 1989; Patterson & Pyle 1991; Reid & Taubenberger 1999).

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Influenza A viruses constantly evolve by the mechanisms of antigenic shift and drift (Webster *et al*. 1992). Consequently, they should be considered as emerging infectious diseases or perhaps as 'continually' emerging pathogens. The importance of predicting the emergence of new circulating influenza strains for subsequent annual vaccine development cannot be underestimated (Gensheimer *et al*. 1999). Pandemic influenza viruses have emerged three times in this century: in 1918 (Spanish influenza, that was an H1N1 subtype strain), in 1957 (Asian influenza, that was an H2N2 subtype strain) and in 1968 (Hong Kong influenza, that was an H3N2 subtype strain) (Webster *et al*. 1992; Cox & Subbarao 2000). How and when novel influenza viruses emerge as pandemic strains is still not understood.

Studying the extent to which the 1918 influenza was like other pandemics may help us understand how pandemic influenzas emerge in general. On the other hand, until and unless we can determine what made the 1918 influenza different from other pandemics, we cannot use the lessons of 1918 for predicting the magnitude of the public health risk that a new pandemic might pose.

2. THE ORIGIN OF PANDEMIC INFLUENZA VIRUSES

The predominant natural reservoir of influenza viruses is thought to be wild waterfowl (Webster *et al*. 1992).

Genetic material from avian strains is periodically transferred to strains that are infectious to humans by a process called reassortment or antigenic shift. Human influenza strains with recently acquired avian surface and internal protein-encoding gene segments were responsible for the pandemic influenza outbreaks in 1957 and 1968 (Scholtissek *et al*. 1978; Kawaoka *et al*. 1989). Since pigs can be infected with both avian and human strains and various reassortants have been isolated from pigs, they have been proposed as an intermediary in this process (Ludwig *et al*. 1995). In 1979, an avian H1N1 influenza A virus (without reassortment) entered the swine population in northern Europe (Ludwig *et al*. 1995) forming a stable viral lineage. Until recently, there was no evidence that a wholly avian influenza virus could directly infect humans, but 18 people were infected with avian H5N1 influenza viruses in Hong Kong in 1997 and six died of complications after infection (Claas *et al*. 1998; Subbarao *et al*. 1998). Although these viruses were very poorly or non-transmissible person to person (Katz *et al*. 1999), their detection indicates that humans can be infected with wholly avian influenza strains. Therefore, it may not be necessary to invoke swine as the intermediary in the formation of a pandemic strain, since reassortment could take place directly in humans.

While reassortment appears to be a critical event in the production of a pandemic virus, a significant amount of data exists to suggest that influenza viruses must also acquire specific adaptations in order to spread and replicate efficiently in a new host. Among other features, there must be functional haemagglutinin (HA) receptor binding and interaction between viral and host proteins (Weis *et al*. 1988). Human-adapted influenza viruses preferentially bind sialic acid receptors with $\alpha(2,6)$ linkages. Those strains adapted to birds preferentially bind $\alpha(2,3)$-linked sugars (Weis *et al*. 1988; Gambaryan *et al*. 1997; Ito *et al*. 1997; Matrosovich *et al*. 1997). Defining the minimal adaptive changes needed for allowing a reassortant virus to function in humans is essential in understanding how pandemic viruses emerge.

Once a new strain has acquired the changes that allow it to spread in humans, its virulence is probably due in large part to the presence of novel surface protein(s), that allow the virus to spread rapidly through an immunologically naive population (Kilbourne 1977). This was the case in 1957 and 1968 and was almost certainly the case in 1918. While immunological novelty may explain much of the virulence of the 1918 influenza, it is likely that additional genetic features contributed to its exceptional lethality. Unfortunately, little is known about how the genetic features of influenza viruses affect their virulence. Virulence (or the degree of illness caused by a particular strain) is complex and involves a number of features, including host factors such as immune status and viral factors such as host adaptation, transmissibility, tissue tropism and viral replication efficiency. The genetic basis for each of these features is not yet fully characterized, but is most probably polygenic in nature (Kilbourne 1977). Sequence analysis of the 1918 influenza virus potentially allows us to address the genetic basis of virulence.

Prior to our preliminary work on the 1918 virus, only two pandemic influenza strains were available for molecular genetic analysis, namely the H2N2 strain from 1957 and the H3N2 strain from 1968. The 1957 pandemic resulted from the emergence of a reassortant influenza virus in which both the HA and neuraminidase (NA) genes had been replaced by gene segments that are closely related to avian strains (Scholtissek *et al*. 1978; Schafer *et al*. 1993; Webster *et al*. 1995). The 1968 pandemic was caused by the emergence of a strain in which the H2 subtype HA gene segment was reassorted with an avian-derived H3 HA gene segment (Scholtissek *et al*. 1978; Webster *et al*. 1995), while retaining the N2 gene segment derived in 1957. It has been shown more recently that the PB1 gene segment was replaced in both the 1957 and 1968 pandemics, in both cases also with a likely avian derivation (Kawaoka *et al*. 1989). The remaining five gene segments, i.e. PA, PB2, nucleoprotein, matrix and non-structural, were all preserved from the H1N1 strains circulating before 1957. These segments were probably the direct descendants of the gene segments present in the 1918 virus. Since only the 1957 and 1968 influenza pandemic strains have been available for sequence analysis, it is not clear what changes are necessary for the emergence of a strain with pandemic potential.

3. HISTORICAL BACKGROUND

The influenza pandemic of 1918 was exceptional in both its breadth and depth. Outbreaks of the disease swept not only North America and Europe, but also spread as far as the Alaskan wilderness and the most remote islands of the Pacific. It has been estimated that 28% of the world's population (500 million people) may have been clinically infected during the pandemic (Frost 1920; Burnet & Clark 1942). The disease was also exceptionally severe, with mortality rates among the infected of over 2.5%, as compared with less than 0.1% in other influenza epidemics (Marks & Beatty 1976; Rosenau & Last 1980). The total mortality that was attributable to the 1918 pandemic ranges from 20–40 million, with the higher number probably being more accurate (Crosby 1989; Patterson & Pyle 1991).

Unlike most subsequent influenza strains, that have developed in Asia, the 'first wave' or 'spring wave' of the 1918 pandemic seemingly arose in the USA in March 1918 (Crosby 1989). However, the near simultaneous appearance of influenza in March–April 1918 in North America, Europe and Asia makes definitive assignment of a geographical point of origin impossible (Jordan 1927). It is possible that a mutation or reassortment occurred in the late summer of 1918, resulting in significantly enhanced virulence. The main wave of the global pandemic, the 'autumn wave' or 'second wave', occurred in September–November 1918. It has been estimated that the influenza epidemic of 1918 killed 675 000 Americans, including 43 000 servicemen who were mobilized for the First World War (Crosby 1989). Its impact was so profound as to depress the average life expectancy in the USA by over 10 years (figure 1) (Grove & Hetzel 1968) and may have played a significant role in ending the First World War conflict (Crosby 1989).

The majority of individuals who died during the pandemic succumbed to secondary bacterial pneumonia, since no antibiotics were available in 1918. However, a subset died rapidly after the onset of symptoms, often

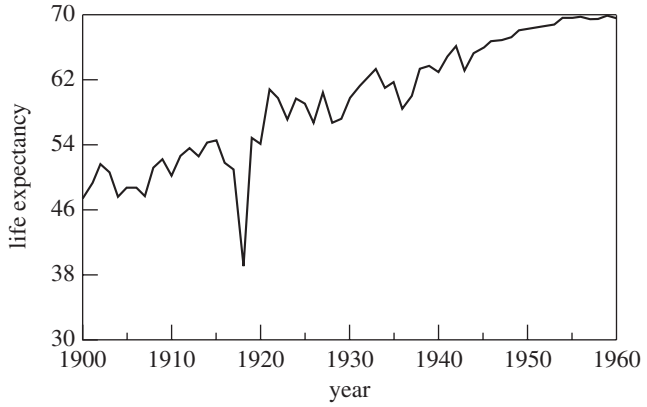

Figure 1. Life expectancy in the USA, 1900–1960, showing the impact of the 1918 influenza pandemic (Linder & Grove 1943; Grove & Hetzel 1968; United States Department of Commerce 1976).

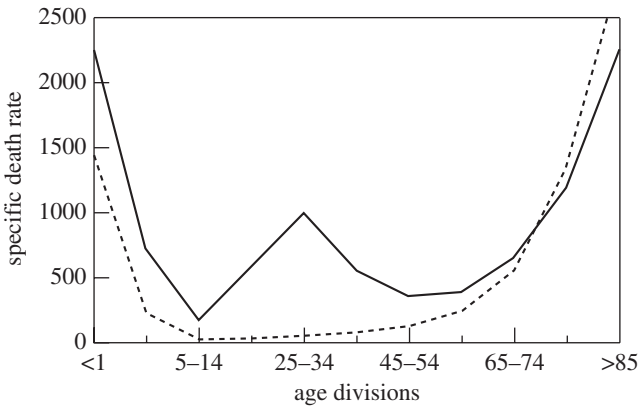

Figure 2. Influenza and pneumonia mortality by age in the USA. Influenza- and pneumonia-specific mortality by age, including an average of the interpandemic years 1911–1915 (dashed line) and the pandemic year 1918 (solid line). The specific death rate is per 100 000 of the population in each age division (Linder & Grove 1943; Grove & Hetzel 1968; United States Department of Commerce 1976).

with either massive acute pulmonary haemorrhage or pulmonary oedema and frequently in less than 5 days (LeCount 1919; Wolbach 1919; Winternitz *et al*. 1920). In the hundreds of autopsies performed in 1918, the primary pathological findings were confined to the respiratory tree and death was due to pneumonia and respiratory failure (Winternitz *et al*. 1920). These findings are consistent with infection by a well-adapted influenza virus that is capable of rapid replication throughout the entire respiratory tree (Reid & Taubenberger 1999; Taubenberger *et al*. 2000). There was no clinical or pathological evidence for systemic circulation of the virus (Winternitz *et al*. 1920).

Furthermore, in the 1918 pandemic most deaths occurred among young adults, a group that usually has a very low death rate from influenza. The influenza and pneumonia death rates for 15–34 year olds were more than 20 times higher in 1918 than in previous years (figure 2) (Linder & Grove 1943; Simonsen *et al*. 1998). The 1918 pandemic is also unique among influenza pandemics in that the absolute risk of influenza mortality was higher in those under 65 years of age than in those over 65 years of age. Strikingly, persons under 65 years old accounted for greater than 99% of all excess influenza-related deaths in 1918–1919 (Simonsen *et al*. 1998). In contrast, the under 65 years age group accounted for 36% of all excess influenza-related mortality in the 1957 H2N2 pandemic and 48% in the 1968 H3N2 pandemic. Overall, nearly half of the influenza-related deaths in the 1918 influenza pandemic were young adults aged 20–40 years (figure 2) (Simonsen *et al*. 1998). Why this particular age group suffered such extreme mortality has not been adequately explained.

The 1918 influenza had the simultaneous infection of both humans and swine as another unique feature. Interestingly, influenza was first recognized as a clinical entity in swine in the autumn of 1918 (Koen 1919), concurrent with the spread of the second wave of the pandemic in humans (Dorset *et al*. 1922–1923). Investigators were impressed by the clinical and pathological similarities in human and swine influenza in 1918 (Koen 1919; Murray & Biester 1930). An extensive review of the diseases of swine by Dimoch (1918–1919), which was published in August 1918, makes no mention of any swine disease resembling influenza. Thus, contemporary investigators were convinced that influenza virus had not circulated as an epizootic disease in swine before 1918 and that the virus spread from humans to pigs because of the appearance of illness in pigs after the first wave of the 1918 influenza in humans (Shope 1936).

Thereafter, the disease became widespread among herds of swine in the US midwest. The epizootic of 1919–1920 was as extensive as in 1918–1919. The disease then appeared among swine in the midwest every year, leading to Shope's (1931) isolation of the first influenza virus in 1930, A/swine/Iowa/30, 3 years before the isolation of the first human influenza virus, A/WS/33, by Smith *et al*. (1933). Classical swine viruses have continued to circulate not only in North American pigs, but also in swine populations in Europe and Asia (Nerome *et al*. 1982; Kupradinun *et al*. 1991, Brown *et al*. 1995).

Severe influenza-like disease outbreaks were not only noted in swine in the USA during the autumn and winter of 1918–1919, but also in Europe and China (Chun 1919; Koen 1919; Beveridge 1977). The classical swine H1N1 lineage became endemic in herds of swine in the USA and there are good data supporting the global circulation of the 1918 influenza virus in pigs concurrent with its circulation in humans. There have been many examples of both H1N1 and H3N2 human influenza A virus strains becoming established in swine since 1918 (Castrucci *et al*. 1993; Brown *et al*. 1998; Zhou *et al*. 2000), while swine influenza A strains have only been sporadically isolated from humans (Gaydos *et al*. 1977; Woods *et al*. 1981).

The unusual severity of the 1918 pandemic and the exceptionally high mortality it caused among young adults have stimulated great interest in the influenza strain that was responsible for this outbreak (Crosby 1989; Monto *et al*. 1997; Kolata 1999). Since the first human and swine influenza A viruses were not isolated until the early 1930s (Shope & Lewis 1931; Smith *et al*. 1933), characterization of the 1918 strain has previously had to rely on indirect evidence (Shope 1958; Kanegae *et al*. 1994). How these archaeserological data relate to

the ongoing effort to sequence the genome of the 1918 virus is discussed below.

4. THE SEROLOGY AND EPIDEMIOLOGY OF THE 1918 INFLUENZA VIRUS

Analyses of antibody titres from 1918 influenza survivors from the late 1930s correctly suggested that the 1918 strain was an H1N1 subtype influenza A virus and was closely related to what is now known as 'classic swine' influenza virus (Shope 1936; Philip & Lackman 1962; Dowdle 1999). The relationship with swine influenza is also reflected in the simultaneous influenza outbreaks in humans and pigs around the world (Chun 1919; Koen 1919; Beveridge 1977). While the historical accounts described above suggest that the virus spread from humans to pigs in the autumn of 1918, the relationship of these two species in the development of the 1918 influenza has not been resolved.

It is not known for certain what influenza A subtype(s) circulated before the 1918 pandemic. In a recent review of the existing archaeserologic and epidemiologic data, Dowdle (1999) concluded that an H3 subtype influenza A strain circulated from the 1889–1891 pandemic to 1918 when it was replaced by the novel H1N1 strain of the 1918 pandemic.

It is reasonable to conclude that the 1918 strain must have contained an HA gene encoding a novel subtype such that large portions of the population did not have protective immunity (Kilbourne 1977; Reid & Taubenberger 1999). In fact, epidemiological data on influenza prevalence by age in the population collected between 1900 and 1918 provide good evidence for the emergence of an antigenically novel influenza virus in 1918 (figure 3) (Jordan 1927). Figure 3 shows two age groups, namely those aged from 5–15 years and those over 65 years. From 1900 to 1917, 11% of total influenza cases in this series were aged between 5 and 15 years, while 6% of influenza cases were in people over 65 years of age. In 1918, the incidence of influenza in those aged between 5 and 15 years jumped to 25% of the total, which is compatible with exposure to an antigenically novel strain, whereas the over 65 years age group accounted for only 0.6% of the influenza cases in 1918. It is likely that this age group accounted for a significantly lower percentage of influenza cases because younger people were so susceptible to the novel strain (as seen in the 1957 pandemic) (Ministry of Health 1960; Simonsen *et al*. 1998), but it is also possible that this age group had pre-existing H1 antibodies. Further evidence for pre-existing H1 immunity can be derived from the age-adjusted mortality data in figure 2. Those individuals over 75 years had a lower influenza and pneumonia case mortality rate in 1918 than they had for the pre-pandemic period of 1911–1917.

A different perspective emerges (figure 4) when 1918 influenza case rates by age (Jordan 1927) are superimposed on the familiar 'W-shaped' mortality curve (as seen in figure 2). As shown, those over 35 years of age in 1918 accounted for a disproportionately high influenza incidence by age, as reflected in the data shown in figure 3. Interestingly, the 5–14 years age group accounted for a large fraction of the 1918 influenza cases (figure 3), but had an extremely low case mortality rate as compared

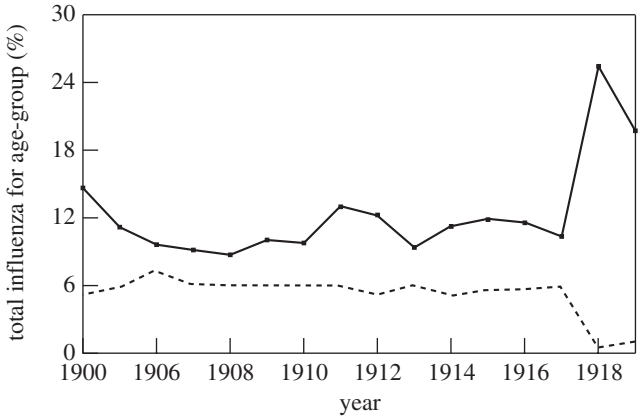

Figure 3. Proportional age distribution of influenza notifications, 1900–1918, Copenhagen, Denmark. Solid line, 5–15 years old age group; dashed line, over 65 years old age group. Adapted from Jordan (1927).

with other age groups (figure 4). Why this age group had such a low case fatality rate cannot currently be explained. Conversely, why the 25–34 years age group had such a high influenza and pneumonia mortality rate in 1918 remains not only enigmatic, but one of the truly unique features of the 1918 influenza pandemic.

Thus, it seems clear that the H1N1 virus of the pandemic contained an antigenically novel haemagglutinin to which both humans and swine were susceptible in 1918. Given the severity of the pandemic, it is also reasonable to conclude that the other dominant surface protein, i.e. neuraminidase, would also have been replaced by antigenic shift before the start of the pandemic (Reid & Taubenberger 1999; Taubenberger *et al*. 2000). In fact, sequence and phylogenetic analyses suggest that the genes encoding these two surface proteins were derived from an avian influenza virus shortly before the start of the 1918 pandemic and that the precursor virus did not circulate widely in either humans or swine before 1918 (Taubenberger *et al*. 1997, 2000; Reid & Taubenberger 1999; Reid *et al*. 1999, 2000). It is currently unclear what other influenza gene segments were novel in the 1918 pandemic virus in comparison with the previously circulating strain. It is possible that sequence and phylogenetic analyses of these gene segments may help to elucidate this question.

5. EVOLUTION OF THE PANDEMIC VIRUS AFTER 1918

After the 1918 pandemic, a concerted effort was made to isolate the causative agent of influenza. Shope isolated the first influenza virus from swine in 1930 (Shope 1931) and Smith *et al*. (1933) isolated the first human influenza virus 3 years later. Since then, a number of archaeserological analyses of age-related haemagglutinin inhibition seroprevalence have been performed using sera collected from the 1930s to the 1960s (Laidlaw 1935; Shope 1936; Davenport *et al*. 1953; Philip & Lackman 1962; Masurel 1976; Dowdle 1999). These analyses have helped to clarify the relationship of the 1918 pandemic virus to the H1N1 viruses that circulated in humans from 1918 to 1957 (which re-emerged in 1977) and in swine since 1918.

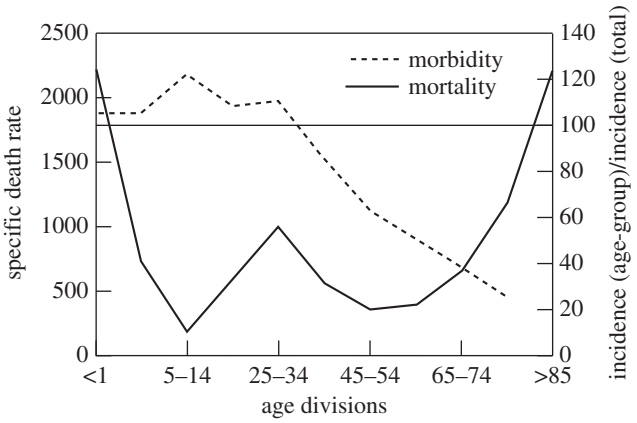

Figure 4. Influenza and pneumonia mortality by age (solid line), with influenza morbidity by age (dashed line) superimposed. Influenza and pneumonia mortality by age as in figure 2. The specific death rate per age group is on the left ordinal axis. Influenza morbidity presented as the ratio of the incidence in persons of each group to the incidence in persons of all ages (= 100) is on the right ordinal axis. The horizontal line at 100 (right ordinal axis) represents the average influenza incidence in the total population. Adapted from Jordan (1927).

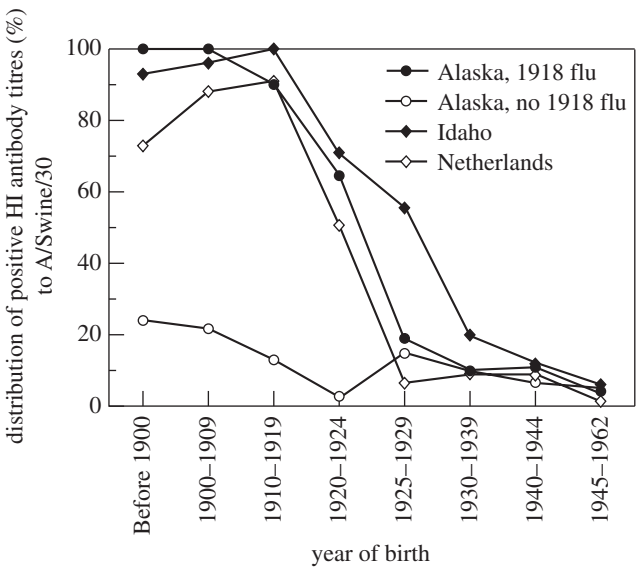

Figure 5. Distribution of positive haemagglutinin inhibition titres to A/Swine/15/30 (H1N1) in human sera collected in 1935–1967. Solid circles, sera collected in Alaskan villages where influenza occurred in 1918; open circles, sera collected in Alaskan villages where no influenza occurred in 1918; solid diamonds, sera collected in Idaho; open diamonds, sera collected in The Netherlands. Adapted from Philip & Lackman (1962), Masurel (1976) and Dowdle (1999).

Whether collected in North America or Europe, the distribution of HI antibody titres to A/Swine/Iowa/30 (H1N1) by birth is remarkably consistent (figure 5). A dramatic increase in A/Swine/Iowa/30 HI antibody titre is seen in individuals born before 1924 and approaches 100% in those born between 1900 and 1918. These data were interpreted by Shope (1936), who concluded that

> swine influenza virus represents a surviving form of the human pandemic virus of 1918, and that it has not had its immunological identity detectably altered by its prolonged sojourn in hogs... (T)he presence in human sera of antibodies neutralizing the swine virus would be considered as indicating that the donors of these sera had undergone an immunizing exposure to or infection with an influenza virus of the 1918 pandemic type (p. 683).

Laidlaw (1935) similarly concluded that 'the virus of swine influenza is really the virus of the great pandemic of 1918, adapted to the pig and persisting in that species ever since' (p. 1118).

These data are most compatible with the model in which the 1918 influenza formed subsequent stable viral lineages in humans and swine that evolved (at least in the antigenic sites) at a higher rate in humans than in swine (Taubenberger *et al*. 2000; Reid *et al*. 2001). Now that the complete genetic sequence of the 1918 virus is being determined, it becomes possible to compare conclusions about the origin and evolution of the 1918 influenza pandemic virus drawn from epidemiological and archaeserological analyses with those drawn from sequence and phylogenetic analyses.

6. GENETIC CHARACTERIZATION OF THE 1918 VIRUS

(a) *Sequence analysis of the HA and NA gene segments*

Frozen and fixed lung tissues from three autumn-wave 1918 influenza victims have been used for examining the genetic structure of the 1918 influenza virus directly. Two of the cases analysed were American army soldiers who died in September 1918, one in Camp Upton, New York and the other in Fort Jackson, South Carolina. The material available consists of formalin-fixed, paraffin-embedded autopsy tissue, haematoxylin- and eosin-stained microscopic sections and clinical histories. A third sample was obtained from an Alaskan Inuit woman who had been interred in permafrost in Brevig Mission, Alaska, since her death from influenza in November 1918. The influenza sequences derived from these three cases have been called A/South Carolina/1/18 (H1N1), A/New York/1/18 (H1N1) and A/Brevig Mission/1/18 (H1N1), respectively (Taubenberger *et al*. 1997; Reid *et al*. 1999, 2000).

Amplification and sequencing of small overlapping RNA fragments extracted from these tissues allowed complete viral gene sequences to be determined for the two surface protein-encoding genes, namely the HA and NA genes (Reid *et al*. 1999, 2000). These sequences confirmed that the 1918 strain was an H1N1 subtype influenza A virus. Although these cases were widely separated geographically, there is very little heterogeneity amongst them at the sequence level, thereby suggesting that the virus was optimally adapted for infecting a majority of the human population in 1918. Only two nucleotide differences in the HA1 domain were noted among the strains (Reid *et al*. 1999).

The sequence of the 1918 HA gene is most closely related to A/Sw/Iowa/30. However, despite this similarity, the sequence has many avian features. Of the 41 amino acids that have been shown to be targets of the immune system and subject to antigenic drift pressure in humans, 37 match the avian sequence consensus, thereby suggesting that there was little immunological pressure on

the HA protein before the autumn of 1918. Another mechanism by which influenza viruses evade the human immune system is the acquisition of glycosylation sites for masking antigenic epitopes. Modern human H1N1s have up to five glycosylation sites in addition to the four found in all avian strains. The 1918 virus only has the four conserved avian sites (Reid *et al*. 1999).

Influenza virus infection requires binding of the HA protein to sialic acid receptors on the host cell surface. The HA receptor-binding site consists of a subset of amino acids that are invariant in all avian HA genes but vary in mammalian-adapted HA genes. To shift from the avian-adapted receptor-binding site configuration (with a preference for α(2,3) sialic acids) to that of swine H1s (which can bind both α(2,3) and α(2,6)) requires only one amino acid change, namely E190D. All three 1918 cases have the E190D change. In fact, the receptor-binding site of one of the 1918 cases (A/New York/1/18) is identical to that of A/Sw/Iowa/30. The other two 1918 cases have an additional change from the avian consensus, namely G225D. Since swine viruses with the same receptor site as Sw/Iowa/30 bind both avian-type and mammalian-type receptors, A/New York/1/18 probably also had the capacity to bind both (Gambaryan *et al*. 1997). The change at residue 190 may represent the minimal change necessary for allowing an avian H1 subtype HA gene to bind mammalian-type receptors (Reid *et al*. 1999), which is a critical step in host adaptation.

The principal biological role of NA genes is the cleavage of the terminal sialic acid residues that are receptors for the HA protein of the virus (Palese & Compans 1976). The active site of the enzyme consists of 15 invariant amino acids that are conserved in the 1918 NA gene. The functional NA protein is configured as a homotetramer in which the active sites are found on a terminal knob carried on a thin stalk (Colman *et al*. 1983). Some early human strains have short (11–16 amino acids) deletions in the stalk region, as do many strains isolated from chickens. The 1918 NA gene has a full-length stalk and has only the glycosylation sites shared by avian N1 strains (Schulze 1997). Although the antigenic sites on human-adapted N1 NA genes have not been mapped, it is possible to align the N1 sequences with N2 subtype NA genes and examine the N2 antigenic sites for evidence of drift in N1. There are 22 amino acids on the N2 protein that may function in antigenic epitopes. The 1918 NA gene matches the avian consensus at 21 of these sites (Colman *et al*. 1983). Thus, like the HA gene, this suggests that the 1918 NA gene had not circulated long in humans before the pandemic (Reid *et al*. 2000).

The 1918 HA and NA genes do not have obvious genetic features that can be directly related to virulence. Two known mutations that can dramatically affect the virulence of influenza strains have been described. HA genes must be cleaved into two pieces, HA1 and HA2, by a host protease for viral activation (Rott *et al*. 1995; Taubenberger 1998). Some avian H5 and H7 subtype viruses acquire a mutation that involves the addition of one or more basic amino acids to the cleavage site, thereby allowing HA gene activation by ubiquitous proteases (Webster & Rott 1987; Kawaoka & Webster 1988). Infection with such a pantropic strain causes systemic disease in birds with near-uniform mortality. This mutation was not observed in the 1918 virus (Taubenberger *et al*. 1997; Reid *et al*. 1999).

The second mutation with a significant effect on virulence through pantropism has been identified in the NA gene of two mouse-adapted influenza strains, A/WSN/33 and A/NWS/33. Mutations at a single codon (N146R or N146Y, leading to the loss of a glycosylation site) appear, like the HA gene cleavage site mutation, to allow the virus to replicate in many tissues outside the respiratory tract (Li *et al*. 1993). This mutation was also not observed in the 1918 virus (Reid *et al*. 2000).

Therefore, neither surface protein-encoding gene has known mutations that would allow the virus to become pantropic. Since clinical and pathological findings in 1918 showed no evidence of replication outside the respiratory system (Wolbach 1919; Winternitz *et al*. 1920), mutations allowing the 1918 virus to replicate systemically would not be expected. However, the relationship of other structural features of these proteins (aside from their presumed antigenic novelty) to virulence remains unknown. The 1918 HA and NA genes are avian-like in their overall structural and functional characteristics, but they also have mammalian-adapted characteristics.

Since virulence cannot yet be adequately explained by sequence analysis of the 1918 HA and NA genes, what can these sequences tell us about the origin of the 1918 virus? The best approach to analysing the relationships between influenza viruses is phylogenetics, whereby hypothetical family trees that take available sequence data and use them for making assumptions about the ancestral relationships between current and historical influenza strains are constructed (Gammelin *et al*. 1990; Fitch *et al*. 1991; Scholtissek *et al*. 1993). Since influenza genes are encoded by eight discrete RNA segments that can move independently between strains by the process of reassortment, these evolutionary studies must be performed independently for each gene segment.

A comparison of the complete 1918 HA and NA genes with those of numerous human, swine and avian sequences demonstrates the following. Phylogenetic analyses based upon HA gene nucleotide changes (either total, synonymous or non-synonymous) or HA gene amino acid changes always place the 1918 HA gene with the mammalian viruses, not with the avian viruses. Synonymous changes place the 1918 HA gene in the human clade (figure 6). Phylogenetic analyses of total or synonymous NA gene nucleotide changes also place the 1918 NA gene sequence with the mammalian viruses, but analyses of non-synonymous changes or amino acid changes place the 1918 NA gene with the avian viruses. Most analyses place the HA and NA genes near the root of the mammalian clade, thereby suggesting that both genes emerged from an avian reservoir just prior to 1918 (Fanning & Taubenberger 1999; Reid *et al*. 1999, 2000; Fanning *et al*. 2000). Clearly, by 1918 the virus had acquired enough mammalian-adaptive changes for functioning as a human pandemic virus and forming a stable lineage in swine.

Identifying the minimal changes necessary for allowing a virus with avian surface proteins to replicate and be transmitted efficiently in mammalian hosts is extremely important for our understanding of the emergence of pandemic influenza viruses. Besides placing viral sequences in their evolutionary context, phylogenetic

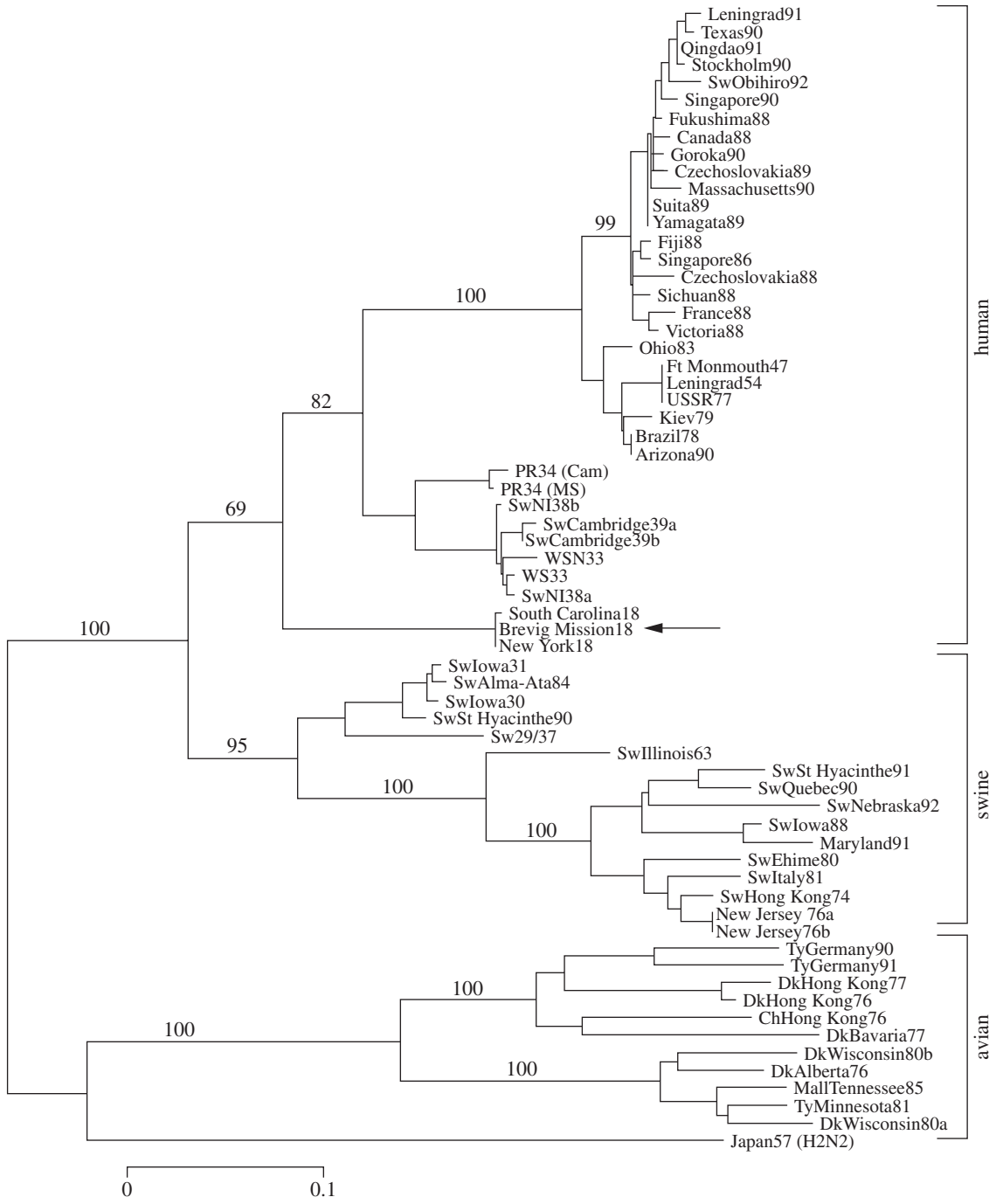

Figure 6. Phylogenetic tree of the influenza virus HA gene segment. Sequences were aligned with LASERGENE software (DNAStar, Inc., Madison, WI, USA) and analysed for phylogenetic relationships by the neighbour-joining method with the proportion of sequence differences as the distance measure (*p*-distance). Synonymous substitutions were analysed and bootstrap values (100 replications) are given for selected nodes. Human, swine and avian clades are identified with brackets. The arrow identifies the position of the 1918 sequences. A distance bar is shown below the tree.

analyses can be used for identifying the features of viral proteins involved in host adaptation. For example, when the 1918 HA gene is compared with strains derived from the independent introduction of an avian H1N1 into European swine in the late 1970s, the E190D change is found in both (Reid *et al*. 1999). Five amino acids in the NA gene were found to change in both. Two of these positions have replacements that are either chemically similar or identical, suggesting that these two sites (residues 285 and 344) may be particularly important in host adaptation of the NA gene (Reid *et al*. 2000).

The accumulation of amino acid changes from a hypothetical ancestral sequence occurs in a linear fashion in mammalian-adapted influenza proteins, with the slope representing the number of amino acid changes per year (Buonagurio *et al*. 1986; Kanegae *et al*. 1994). Such a regression analysis is shown for human and swine H1 and N1 proteins using available strains from 1930 to the present and excluding the 1918 sequence (Taubenberger *et al*. 2000) (figure 7). When the 1918 HA and NA gene data points are then placed on the graph they are located close to the hypothetical common ancestor of human and

swine H1N1 strains. The *x*-intercept of the lines suggests that the surface protein-encoding genes of the virus may have entered mammals just prior to the 1918 pandemic. Together, the phylogenetic analyses support the conclusion that the sequences derived from the 1918 cases are very similar to those of the hypothetical common ancestor of both human and swine H1N1 strains. The archaeserological data also support this same conclusion and, further, both support the conclusion that the 1918 HA gene was not 'swine like' but rather that A/Swine/Iowa/30 was still much more '1918 like' than concurrently circulating human influenza strains because of the enormous antigenic drift pressure exerted on the human H1N1 lineage from 1918 to the 1930s (Laidlaw 1935; Shope 1936; Dowdle 1999).

(b) *Sequence analysis of the non-structural gene segment*

The complete coding sequence of the 1918 non-structural (NS) segment was recently completed (Basler *et al*. 2001). The functions of the two proteins NS1 and NS2 (nuclear export proteins, NEP), that are encoded by overlapping reading frames (Lamb & Lai 1980) of the NS segment, are still being elucidated (Garcia-Sastre *et al*. 1998; Li *et al*. 1998; O'Neill *et al*. 1998). The NS1 protein has been shown to prevent type I interferon (IFN) production by preventing activation of the latent transcription factors IRF-3 (Talon *et al*. 2000) and NF-κB (Wang *et al*. 2000). One of the distinctive clinical characteristics of the 1918 influenza was its ability to produce rapid and extensive damage to both the upper and lower respiratory epithelium (Winternitz *et al*. 1920). Such a clinical course suggests a virus that replicated to a high titre and spread quickly from cell to cell. Thus, an NS1 protein that was particularly effective at blocking the type I IFN system might have contributed to the exceptional virulence of the 1918 strain (Garcia-Sastre *et al*. 1998; Talon *et al*. 2000; Wang *et al*. 2000). In order to address this possibility, transfectant A/WSN/33 influenza viruses were constructed with the 1918 NS1 gene or with the entire 1918 NS segment (coding for both NS1 and NS2 proteins) (Basler *et al*. 2001). Viruses containing 1918 NS genes were attenuated in mice compared with wild-type A/WSN/33 controls in both cases. The attenuation demonstrates that NS1 is critical to the virulence of A/WSN/33 in mice. The 1918 NS1 gene differs from that of the WSN gene at 10 amino acids. The amino acid differences between the 1918 and A/WSN/33 NS segments may be important in the adaptation of the latter strains to mice and probably account for the observed differences in virulence in this set of experiments. Thus, further experiments using different viral and animal backgrounds may be necessary for testing the hypothesis that the 1918 NS1 gene specifically contributed to virulence.

The entire 1918 NS segment coding sequence (838 nucleotides) was determined from the frozen sample obtained from Brevig Mission, Alaska (A/Brevig Mission/1/18 (H1N1)). Phylogenetic analysis using neighbour joining of 63 NS1 nucleotide sequences produced a tree with nine clades: avian 1–5, equine 1 and 2, human and swine. Neighbour joining of 61 NEP nucleotide sequences produced a tree with the same nine clades seen in the NS1 tree. The A/Brevig Mission/1/18 NS gene was within and near the root of the swine clade for NS1 and within and near the root of the human clade for NS2 (NEP) (Basler *et al*. 2001).

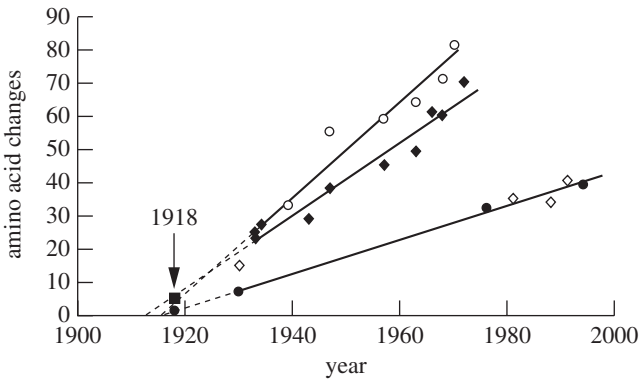

Figure 7. Changes in the HA and NA proteins over time. The number of amino acid changes from a hypothetical ancestor was plotted versus the date of viral isolation for viruses isolated from 1930 to 1993. Open circles, human HA gene; solid diamonds, human NA gene; solid circles, swine HA gene; open diamonds, swine NA gene. Regression lines were drawn, extrapolated to the *x*-intercept and then the 1918 data points for the 1918 HA gene (solid squares) and 1918 NA gene (solid circles) were added to the graph (arrow).

7. CONCLUSIONS AND FUTURE WORK

Three of the eight gene segments of the 1918 influenza virus have been sequenced and analysed. They have shed light on the origin of the virus and strongly support the hypothesis that the 1918 virus was the common ancestor of both subsequent human and swine H1N1 lineages. Sequence analyses of the genes to date offer no direct clue as to the exceptional virulence of the 1918 strain. However, they have shown that several hypotheses as to the virulence of the 1918 virus were incorrect (Goto & Kawaoka 1998).

Whether any particular genetic features of the virus can be directly related to its exceptional virulence is as yet unclear. In future work it is hoped that the 1918 pandemic strain can be placed in the context of the influenza strains that preceded and followed it. The direct precursor of the pandemic virus, the first or spring-wave strain, lacked the exceptional virulence of the autumn-wave strain. Identification of an influenza RNA-positive case from the first wave would have tremendous value in deciphering the genetic basis for virulence by allowing differences in the sequences to be highlighted. Identification of pre-1918 human influenza RNA samples would clarify which gene segments were novel in the 1918 virus.

In many respects, the 1918 influenza pandemic was similar to other influenza pandemics. The pandemic was generally different in degree but not in kind from previous and subsequent pandemics in its epidemiology, disease course and pathology. However, there are some characteristics of the pandemic that appear to be unique. Mortality was exceptionally high, ranging from five to 20 times higher than normal. Clinically and pathologically, the high mortality appears to be the result of a higher

proportion of severe and complicated infections of the respiratory tract without systemic infection or involvement of organ systems outside the normal targets of influenza viruses. The mortality was concentrated in an unusually young age group. Finally, the waves of influenza activity followed on each other unusually rapidly, resulting in three major outbreaks within the duration of a year. Each of these unique characteristics may find their explanation in genetic features of the 1918 virus. The challenge will be in determining the links between the biological capabilities of the virus and the known history of the pandemic.

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