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Antigenic Complementarity between Influenza A Virus and *Haemophilus influenzae* may Drive Lethal Co-Infection Such as that Seen in 1918-19 Pandemic

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Abstract

Pfeiffer's bacillus, now known as *Haemophilus influenzae* (HI), was strongly implicated in the high lethality of the 1918-19 influenza pandemic. Influenza virus (IV) infection is often complicated by (HI) super-infection and certainly was so in 1918-19. We propose that the influenza pandemic of 1918-19 was caused by concurrent pandemics of IV and HI that overwhelmed co-infected individuals through antigenic synergy between the two infections. Using T cell receptor sequences specific for IV and HI antigens, we demonstrate that IV and HI antigens induce molecularly complementary TCR responses. Additionally, IV antibody (multiple strains) precipitates and co-neutralizes HI antibody. HI antibody inhibits IV antibody binding to IV antigens. Human T cell receptor sequences generated against the hemagglutinin antigen of IV mimic multiple HI antigens, most notably the outer membrane protein P1, which is recognized by HI antibody and binds to IV. Antigenic complementarity between HI and IV thereby produces complementary antibodies that neutralize each other. Complementarity may also permit IV to "piggyback" on HI so that co-infection risk increases. The combined HI-IV theory of pandemic influenza also explains the odd epidemiology of the Great Pandemic. Uncomplicated influenza generally has a "U" shaped mortality curve, in which the very young and the very old at are at greatest risk of dying, Oddly, the 1918-1919 influenza pandemic is unique in the history of such pandemics in having a "W" shaped mortality curve in which 20-40 year old people were at high risk of dying. This "W" shaped mortality curve has never been explained. Notably, the incidence of HI infections often displays a "W" shaped curve, in which 20 to 45 year olds are at highest risk of invasive disease. This explanation of the Great Pandemic has implications for surveillance, treatment and prevention of any future influenza pandemic. Surveillance must include not just IV variants but possible bacterial co-infections such as HI. Vaccination against HI, and other bacterial superinfections of IV, may be the most effective way to prevent lethal sequelae in influenza pandemics. Further, antibiotics – not available during the Great Pandemic – may be key elements in preventing deaths during any future pandemic. Our results also warn against delivering HI and IV vaccinations simultaneously.

Keywords

T cell receptors; Antibody complementarity; Antigenic complementarity; Synergistic infections; Idiotype-antiidiotype; Viral-bacterial synergism; W-shaped mortality curve; Pandemic influenza; Bacterial influenza; Cytokine storm; Influenza epidemiology

Introduction

A rapidly fatal swine influenza in hogs was first recognized in 1918 concomitantly with the emergence of the pandemic influenza that affected human beings and the two were considered by experts at that time to be identical [1,2]. Viruses were first isolated only in 1921, so their role in swine and human influenzas was at best hypothetical. Many bacteriologists attributed the pandemic flu to Pfieffer's bacillus (*Haemophilus influenzae*), which was isolated in the majority of cases [3-5]. When highly contagious, rapidly fatal swine influenza re-emerged in hogs in 1928-29, Shope saw an opportunity to elucidate the causes of the 1918-19 human pandemic as well, isolating several closely related viruses from the hog epidemic [1]. His 1931 paper is often cited as the first substantial evidence that influenza is caused by a virus. Shope, however, was puzzled by his results. The course of the experimental disease caused by these viruses was much milder than that found in either the hog epidemic or the human pandemic, and was never fatal, a result confirmed by MacBryde et al. [6]. So Shope looked for another agent, isolating from all of the fatal hog cases Pfieffer's bacillus (*H. influenzae*) [7]. Shope found, however, that *H. influenzae* was just as incapable as his virus of reproducing the pathology and mortality of either the hog epidemic or the human pandemic [7]. Hogs infected with his *H. influenzae* rarely even became ill.

Having found both a virus and a bacterium in all of the fatal cases of swine flu, and having failed to reproduce the fatal disease with either infectious agent by itself, Shope decided to expose hogs to both simultaneously [8]. He found that all such dual-infected hogs developed a disease of extremely rapid onset that was almost 100% fatal within several days. Symptoms mimicked exactly the pathology associated with both the 1928 hog epidemic and the rapid fatalities described in the 1919 human pandemic, including very high fever, railing cough, sputum expectoration, coagulopathies, hemorrhage, and cyanosis [8]. Moreover, pigs previously exposed to *H. influenzae* were immune to the fatal combined infection, as were those previously exposed to the influenza virus [8]. Infection with *H. influenzae* did not, however, prevent infection with influenza virus, or *vice versa*. Shope concluded that, "since the influenza-like bacillus is always found in the field and experimental cases, and is capable experimentally of converting the mild disease caused by the filtrate [virus] into clinically and pathologically typical swine influenza, it seems probable that both the filterable agent [virus] and the bacillus are etiologically essential to the production of the disease and that, in this role, they act synergistically [9]."

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Despite the fact that Shope's results were subsequently confirmed by Bang [10] in chick embryos with human strains of virus and bacterium, the proposition that rapidly fatal epidemic and pandemic swine influenzas are due to synergistic viral and bacterial infections has almost completely been ignored until very recently. One reason may be the predominance of the "one disease, one agent" dogma in modern etiological reasoning. Another may be the inconsistent and confusing definitions of an influenza diagnosis that, since before the 1918-19 pandemic, has referred to a constellation of clinical symptoms including anything from a brief, non-fatal, self-resolving disease consisting mainly of fever and headache to more severe symptoms that ranged from a railing cough, sputum expectoration, hemorrhage, cyanosis, and rapid death [4]. To some extent, the terminological confusion has persisted to this day, in part due to the fact that the rapidly fatal forms of the pandemic influenza of 1918-19, and of more recent avian influenzas, are described using the same terminology as the non-fatal influenzas studied by Smith et al. [11] in their isolation of the influenza virus in the early 1930s and the everyday disease that afflicts millions each year.

Influenza viruses cause what we today call (uncomplicated) influenza, which is very rarely fatal in immunocompetent individuals and we do not question that proven etiology. That said, it is worthwhile to revisit Shope's synergistic experiments in order to question whether rapidly fatal forms of human and swine "influenza" are simply unusual mutations of the normal annual forms of the virus [12], or whether Shope may have been correct in asserting that the rapidly fatal human and hog forms of the disease are actually synergistic diseases that may require specific antigenic interactions between a mutated virus and a particular strain of bacterium. We are stimulated in this undertaking by the recent work of Brundage [13,14], who has argued that the epidemiology of the 1918-1919 pandemic influenza can be understood only in light of viral-bacterial interactions and also by Morens et al. [15] finding that bacterial co-infections were present in all fatal cases that have been autopsied from that 1918-19 pandemic.

We hypothesize that infections that interact synergistically involve antigens that are molecularly complementary. Complementary antigens will induce complementary (or idiotype-antiidiotype) immune responses that confuse antigen and antibody [16,17]. We further propose that this antigenic complementarity will be reflected in the T cell receptor (TCR) repertoire that is activated during infection. A TCR specific for one infectious agent antigen may mimic the antigenicity of a second, complementary infectious agent. TCR sequences may therefore provide clues as to the specific antigenic interactions that may underlie disease agent synergism. While these hypotheses may seem unconventional, their utility and fruitfulness will be justified by the experimental tests of them reported below and the further testable predictions we can draw from them. We also believe that our synergistic infection hypothesis can explain the unusual "W"-shaped mortality curve of the 1918-1919 influenza pandemic, which was unique to that influenza outbreak.

Materials and Methods

Similarity searching

T-cell receptor (TCR) V-beta-D-J-beta sequences are complementary to their antigens. If, as we have proposed here, influenza viruses and *H. influenzae* are complementary antigens, then it would follow that TCR against influenza virus might mimic some proteins on *H. influenza*. Thus, TCR specific for influenza virus proteins, including haemagglutinin H3N2 protein 306-318 PKYVKQNTLKLAT [17] (Table 1) were used to screen the SwissProt/TREMBL database using the BLAST similarity search algorithm for bacterial proteins that might mimic those IV TCR. The resulting similarities were then screened for their relevance to human respiratory disease. The most disease-relevant and statistically significant sequence identified was *H. influenzae* Outer Membrane P1 143-163: SEYDDSYDAGIFGGKTDLSAI; Table 1. This HI sequence was then entered into BLAST to determine the extent to which it is shared by other bacteria (Table 2). Similarly, the influenza virus haemagglutinin H3N2 peptide sequence 306-318 was entered into

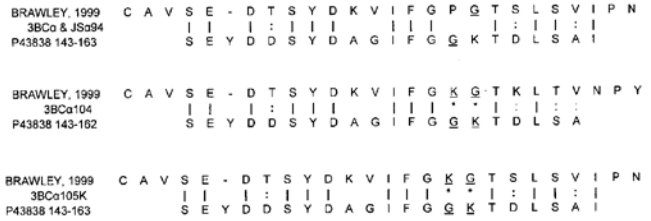

Table 1: TCR Anti-Haemagglutinin Similarity to H. Influenzae OMP. Results of a BLAST search of the SwissProt database looking for the best similarities between bacterial proteins and T cell receptor (TCR) sequences selected for binding to influenza virus (IV) hemagglutinin H3N2 peptide 306-318 (18). The best match in all cases was *Haemophilus influenzae* outer membrane protein residues (OMP) 143-163, which is in an extracellular region of the protein according to the SwissProt accession data. The best match is 13 identities out of 21 amino acids (62%) with three to five additional conserved substitutions. Because the TCR used for similarity searching is complementary to IV haemagglutinin, it is assumed that HI OMP is also likely to be complementary to IV haemagglutinin.

Klebsiellia pneumoniae long-chain fatty acid outer membrane transport protein (FADL) B5XVV9	134 EYNDDYAAGSMGGKTDL	150
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAG I FGGKTDLSAI	163
Neisseria meningitidis outer membrane protein A [OMPA] Q51240, P57041, P13415	342 NTSYDQ I IAGVDYDFS	357
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAG IFGGKTDLSAI	163
Neisseria meningitides outer membrane protein A [OMPA] Q51240, P57041, P13415	146 SNNDVASQLG IFKRHDDM	164
Haemophilus parasuis Long-chain fatty acid transport protein p1/47 kDa outer membrane protein [OMPP1] B8F340	141 SEFP TNYSAGVFAGKTELTAV	161
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAG IFGGKTDLSAI	163
Actinobacillus pleuropneumoniae Putative outer membrane protein [OMPP1] B3GXS8	129 SEYPDDYNAGSIGGKTELQA	148
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAG IFGGKTDLSAI	163
Pasteurella multocida 47 kDa outer membrane protein OMP47 P80603	129 EFDDKYAGGFLGGKTDLTAI	148
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAG I FGGKTDLSAI	163
Escherichia coli long-chain fatty acid outer membrane transport protein (FADL) B7MXM2	133 EFTDNYAAGIYGGKTDLQTI	152
Haemophilus influenzae outer membrane p1	143 SEYDDSYDAGIFGGKTDLSAI	163

Table 2: OMP Homologies Shared by Bacteria Complicating Influenza. A BLAST search reveals that many bacteria associated with complications of influenza infections have outer membrane protein regions that are very similar to that of the HI complement to IV identified in Table 1. These similarities suggest that these additional bacterial proteins may also be complementary to IV hemagglutinin.

BLAST to determine the extent to which it is shared by other viruses (Table 3).

Peptide synthesis

The influenza virus haemagglutinin H3N2 peptide 306-318 PKYVKQNTLKLAT and the *H. influenzae* Outer Membrane P1 143-163 peptide SEYDDSYDAGIFGGKTDLSAI, as well as an assortment of cytomegalovirus and Streptococcal peptides were synthesized in milligram quantities and purified to >99% purity (according to HPLC) by the Mass Spectrometry, Synthesis and Structure Facility of the Biochemistry Department of Michigan State University. Influenza A H1N1 Hemagglutinin 96-106 (YPYDVPDYA) – a sequence bearing no similarities to sequences identified in the similarity searches described above – was obtained from Sigma-Aldrich.

Influenza vaccines

Two influenza vaccines were used in these experiments: Flulaval 2007/2008 Vaccine (Glaxo Smith Kline) containing hemagglutinin from A/Solomon Islands/3/2006 (H1N1), A/Wisconsin, 67/2005 (H3N2), and B/Malaysia/2506/2004 and Fluogen Influenza A and B Vaccine 1987/1988 (Parke Davis) containing hemagglutinin from A/Taiwan/1/86 (H1N1), A/Leningrad/360/86 (H3N2), and B/Ann Arbor/1/86).

Antibodies

The antibodies used in these experiments were as follows: Influenza A Virus Hemagglutinin 18D5: Mouse Monoclonal, sc-66144 from Santa Cruz Biotechnology; Influenza A Virus Hemagglutinin F8: Mouse Monoclonal, sc-52026 from Santa Cruz Biotechnology; Rabbit anti *H. Influenzae*: #B47822R, from Biodesign International; Goat anti Influenza B Virions HRP: #B65344G from Biodesign International; Goat anti Influenza A HRP: #B65314G from Biodesign International; Goat anti Influenza A: #B65311G from Biodesign International; Goat anti Influenza A: #B65141G from Biodesign International; Goat anti Influenza A (H1N1): #B65243G from Biodesign International; Goat anti Influenza A (H3N2): #B65314G from Biodesign International.

H1 320-332 (SWISSPROT P03452)	PKYVRSAKLRMVT
H3 306-318	PKYVKQNTLKLAT
H2 317-329 (SWISSPROT Q05JH5)	PKYVKSE RLVLAT
H4 320-332 (SWISSPROT A1ILQ9)	PKYVKQGSLKLAT
H3 306-318	PKYVKQNTLKLAT
H6 319-331 (SWISSPROT A0A3I8)	PKYVNVKSLKLAT
H5 319-331 (SWISSPROT Q77XR3)	PKYVKSNRLVLAT
H3 306-318	PKYVKQNTLKLAT
H5 319-331 (SWISSPROT Q2Z1U6)	PKYVKSDKLVLAT
H7 315-327 (SWISSPROT P26098)	PRYVKQKSLMLAT
H3 306-318	PKYVKQNTLKLAT
H9 297-308 (SWISSPROT Q8QZ87)	PKYVGVKSLKLA

Table 3: Conservation of Haemagglutinin Region Complimentary to *H. Influenzae* OMP. Randomly selected results of an LALIGN search demonstrating that the IV hemagglutinin sequence (H3N2 peptide 306-318) (18) used in Table 1 is very highly conserved among all strains of influenza virus. Approximately 75 sequences were investigated. Those shown here illustrate the *greatest variations*. Most HI hemagglutinin sequences are identical to H3N2 peptide 306-318.

UV experiments

The *H. influenzae*, influenza hemagglutinin, CMV, and streptococcal peptides listed above were made up into stock solutions at 1.0 mg/ml (ca. 1.0 mM) in pH 7.4 phosphate buffer (Fisher Scientific). One of the peptides was then diluted to 10 μM using pH 7.4 buffer solution, and 100 uL of this peptide solution added to a series of wells in a crystal 96-well plate. Another peptide was serially diluted by thirds from its stock solution, again using pH 7.4 buffer solution. 100 μL of each dilution of the second peptide was added to one of the wells in the crystal plate already containing the first peptide, and 100 μL of each dilution of the second peptide was also added to a new well in the crystal plate. 100 μL of buffer was added to each of the wells containing a dilution of the second peptide. Two control wells were also set up: one contained 200 μL of buffer; the other 100 μL of buffer plus 100 μL of the first peptide. Ultraviolet (UV) spectra from 190 through 260 nm of each well were obtained using a Spectramax Plus scanning spectrophotometer and the data gathered using SoftMax Pro 4.0 software. These curves were examined for the absorbance at which the greatest spectral shifts occurred (usually between 200 and 225 nm) and these data analyzed to produce binding constants (Kd) determined by the difference in absorbance between the absorbance obtained from the mixture of the peptides (minus buffer) and the sums of each peptide alone (minus buffer) at each peptide concentration. Data were analyzed and plotted using Excel. Binding constants were determined by locating the inflection point of the binding curve, if there was one. Because of the concentrations utilized, binding constants greater than 10^{-3} M could not be measured.

ELISA

The ability of the antibodies against *H. influenzae* and influenza virus listed above to recognize the peptides synthesized for this study was tested using enzyme-linked immunoadsorption assay (ELISA). Each peptide was serially diluted as described for the UV spectrophotometric studies and 100 μl of each serial dilution added to a round-bottomed ELISA plate (Costar), incubated for an hour, and then triply washed with a 1% solution of TWEEN 20 in phosphate buffer. 200 μl of a 2% polyvinylalcohol (PVA) blocking agent in phosphate buffer was added to each well, incubated for an hour, and triply washed. 100 μl of a 1:100 dilution of an antibody was then added to each well, incubated for an hour, and triply washed. 100 μl of a 1/1000 dilution of a horse-radish-peroxidase (HRP) linked secondary antibody (Chemicon) appropriate to the species of the antibody was then added to each well and incubated for an hour before being washed out. Finally, 100 μl of ABTS single reagent (Chemicon) was added to each well, incubated for 30 minutes, and the plate read at 405 nm in a Spectromax Plus scanning spectrometer. All combinations and controls were run in duplicate and the resulting values averaged.

Antibody-antibody binding experiments (da-elisas)

Since we hypothesized that *H. influenzae* and influenza virus antigens may be antigenically complementary to each other, the antibodies listed above were tested for their propensity to bind to each other (i.e., for their ability form idiotype-antiidiotype pairs) using a double-antibody enzyme-linked immunoadsorption assay

DA- ELISA protocol (55). DA-ELISA is performed just as a standard ELISA is, but the antigen is replaced with a primary antibody (plated in dilutions from 1/30 through 1/1,000,000 by thirds in pH 7.4 phosphate buffer). 100 µl of each serial dilution of the primary antibody was added to an ELISA plate (Costar), incubated for an hour, and then triply washed with a 1% solution of TWEEN 20 in phosphate buffer. 200 µl of a 2% polyvinylalcohol (PVA) blocking agent in phosphate buffer was added to each well, incubated for an hour, and triply washed. 100 µl of a 1:100 dilution of a second antibody was then added to each well, incubated for an hour, and triply washed. 100 µl of a1/1000 dilution of a horse-radish-peroxidase (HRP) linked tertiary antibody (Chemicon) appropriate to the species of the second antibody was then added to each well and incubated for an hour before being washed out. Finally, 100 µl of ABTS single reagent (Chemicon) was added to each well, incubated for 30 minutes, and the plate read at 405 nm in a Spectromax Plus scanning spectrometer. All combinations and controls were run in duplicate and the resulting values averaged. Note that the primary and test (second) antibodies must be from different species in order for the reporter (enzyme-linked) antibody to be able to differentiate between them. Thus, not all possible combinations of all antibodies can be tested using this procedure.

Competitive ELISAs

One consequence of antigenic complementarity between *H. influenzae* and influenza virus antigens might be that the resulting complementary antibodies would interfere with antibody binding to their primary antigen. Thus, competitive ELISAs were run in which the *H. influenzae* and influenza virus antibodies were premixed and allowed to incubate prior to being applied to the ELISA plate. Beginning at a 1:10 concentration, serial dilutions by thirds were made of goat influenza A antibodies (goat anti Influenza A (H1N1) #B65243G from Biodesign International; Goat anti Influenza A (H3N2) #B65314G from Biodesign International). 100 ul of each influenza A antibody dilution was mixed with 100 µL of rabbit *Haemophilus influenzae* antibody (rabbit anti *H. influenzae* #B47822R, from Biodesign International) at a 1:100 concentration. An identical set of goat influenza A antibody serial dilutions mixed with buffer instead of *H. influenzae* antibody were made at the same time. These combinations were incubated at room temperature for two hours during the set-up of the ELISA. To set up the ELISA plates, 100 µL of Fluogen or Flulaval influenza vaccine at a 1:100 dilution in pH 7.4 buffer was added to a series of wells on Costar ELISA plates. The plates were shaken for an hour, washed, and 200 µL of PVA was added to the wells. After the plates were shaken for another hour and washed, goat influenza A antibody pre-mixed with buffer or goat influenza A antibody pre-mixed with rabbit *Haemophilus* antibody was added to each well. Following another hour of being shaken, HRP-labeled antibody against goat was added at a 1:1000 concentration. The HRP-antibody was washed out and 100 µL of ABTS added to each well. The plate was read at 405 nm 30 minutes later.

Results

Several T cell receptors (TCR) specific for influenza virus haemagglutinin H3N2 protein 306-318 PKYVKQNTLKLAT were isolated by Brawley and Concannon [18]. If the synergy between influenza virus and *H. influenzae* is due to the complementarity of their antigens, then one would expect these anti-haemagglutinin TCR sequences to mimic antigens found in *H. influenzae*. Table 1 shows the results of a BLAST similarity search of the entire SwissProt protein database of bacterial proteins against TCR sequences known to be reactive against the influenza virus haemagglutinin H3N2 protein 306-318 PKYVKQNTLKLAT [18]. The most significant similarities are the three shown in Table 1, in which there is 62% identity over a sequence of 21 amino acids with additional conserved substitutions. All three sequences are matches to the *H. influenzae* Outer Membrane protein, P1 143-163: SEYDDSYDAGIFGGKTDLSAI. Other infectious agents associated with super-infection of IV that appeared in the BLAST search are shown in Table 2. These other infectious agents include *K. pneumoniae, N. meningitides, A. pleuropneumoniae*, and *P. multocida.*

Table 3 shows the results of a BLAST similarity search comparing the influenza virus haemagglutinin H3N2 protein 306-318 PKYVKQNTLKLAT sequence [18] with the SwissProt viral database. The search revealed that this particular region of the haemagglutinin protein is highly conserved among all serotypes of IV, and a random sample from the hundreds of sequences evaluated is shown in Table 3.

In addition, a BLAST similarity search comparing the *H. influenzae* Outer Membrane protein, P1 143-163 sequence SEYDDSYDAGIFGGKTDLSAI to the SwissProt bacterial database demonstrated that this sequence is almost perfectly conserved (with only a few single amino acid substitutions) across all sequenced strains of *H. influenzae,* as well as *H. influenzae suis* and other related *Haemophilus* species. These data are not shown in tabulated form as there are so few variants as to make such a table uninformative.

Since TCR are complementary to their antigens, and the *H. influenzae* Outer Membrane Protein P1 143-163 is highly similar to TCR against IV haemagglutinin, we hypothesized that the HI Outer Membrane protein would also be complementary to IV haemagglutinin. In order to determine whether this logical inference is correct, these two peptides were synthesized and their possible binding investigated using UV spectrophotometry. Figure 1 shows the resulting binding curve determined at 200 and 225 nm. The 200 nm binding constant is approximately 6.5 x 10^{-7} M. The binding constant at 225 nm is about 1.6 x 10^{-7} M. Table 4 shows a selection of additional

Table 4: Binding Constants for Haemagglutinin-OMP Interaction and Controls. Results of U. V. spectroscopic binding studies at 215 nm between HI outer membrane protein 143-163 and various bacterial protein sequences: IV H3N2 hemagglutinin 306-318; H1N1 hemagglutinin 96-106; cytomegalovirus UL56 723-739; and group A streptococcal protein O50278 6-19. The peptide sequences used are listed in the second column. Binding constants (M) are provided in the table. The binding of IV H3N2 306-318 to HI OMP 143-163 was 0.9 µM, several orders of magnitude better binding than any of the other combinations tested. See Figure 1 for additional binding data at 200 nm.

PEPTIDE		H1 OMP1	H3N2 306	H1N1 96	CMV
***H. Influenzae* OMP1 143-163**	SEYDDSYDAGIF-GGKTDLSAI				
Influenza A H3N2 Hemagglutinin 306-318	PKYVKQNTLKLAT	9.0 x 10^{-7}			
Influenza A H1N1 Hemagglutinin 96-106	YPYDVPDYA	1.1 x 10^{-4}	> 10^{-3}		
Cytomegalovirus UL56 723-739	RDELHPDRD-VILTYNKE	1.3 x 10^{-4}	2.5 x 10^{-4}	1.3 x 10^{-4}	
GAS O50278 6-19	ALTVLGAGFAN-QTE	1.7 x 10^{-4}	> 10^{-3}	1.7 x 10^{-4}	> 10^{-3}

controls that involved the binding of the HI peptide with another IV haemagglutinin peptide and other viral and bacterial peptides derived from cytomegalovirus (CMV) and group A streptococcus (GAS) proteins, as well as tests for binding between these control peptides. The binding of the HI outer membrane peptide to the IV haemagglutinin peptide is several orders of magnitude tighter than any of the controls. These data confirm specific complementarity between the IV haemagglutinin peptide identified by Brawley and Concannon [18] and the HI outer membrane peptide identified by similarity searching using the TCR sequences against this IV haemagglutinin peptide [18].

It follows logically from the data in Table 4 and Figure 1 that the IV haemagglutinin peptide and the HI outer membrane peptide may be antigenically complementary as well as physically complementary. That is to say, antigens that bind to each other with stereochemical specificity should result in antibodies that bind to each other with stereochemical specificity. A test of this inference is to determine whether antibodies against IV recognize the IV haemagglutinin peptide; whether antibody against HI recognizes the outer membrane peptide; and if both occur, whether antibodies against IV react to antibodies against HI as if they were idiotype-antiidiotype pairs. In this case, molecular complementarity would be mirrored in antigenic complementarity resulting in antibody complementarity. This is, in fact, what we observed.

Figure 2 shows that rabbit polyclonal antibody against HI recognizes the HI outer membrane peptide as an antigen and that two monoclonal antibodies against IV haemagglutinin recognize the IV haemagglutinin peptide. Not shown is data that the polyclonal antibodies used in these studies also recognize the IV haemagglutinin peptide. Correspondingly, Figure 3 shows that the HI antibody binds to two polyclonal antibodies against IV and Figure 4 shows that HI antibody binds to the two monoclonal antibodies against the IV haemagglutinin peptide that were used in Figure 2. Figures 2 and 4 show some of the controls that were performed as part of these experiments. HI antibody did not recognize polyclonal antibody against influenza virus type B, *Staphylococcus aureus*, or *Streptococcus pneumoniae*. Additional controls are summarized in Table 5.

Finally, if antibody against HI binds with significant affinity to antibody against IV, then antibody against IV should be impaired in its ability to recognize its antigen. To test this consequence, we obtained binding curves for IV antibodies against two influenza vaccine mixtures, Flulaval and Fluogen. IV antibodies at the same concentrations were then mixed with a constant concentration of HI antibody, the mixtures were allowed to incubate at room temperature for two hours, and these mixtures were then tested for their ability to recognize the Flulaval and Fluogen vaccines. Figure 6 shows that the presence of HI antibody mixed with the IV antibody decreases the affinity of IV antibodies for the Flulaval vaccine by about a third of a log unit. Identical results were obtained for binding to Fluogen vaccine (data not shown).

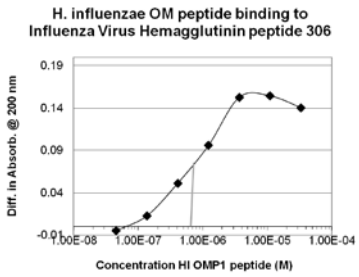

Figure 1: Results of U. V. spectroscopic binding study of HI OMP 143-163 peptide to IV H3N2 306-318 peptide at 200 nm showing that the binding constant is approximately 6.0 x 10-7 M. The data reported in Table 4 are at 215 nm and the calculated binding constants differ insignificantly.

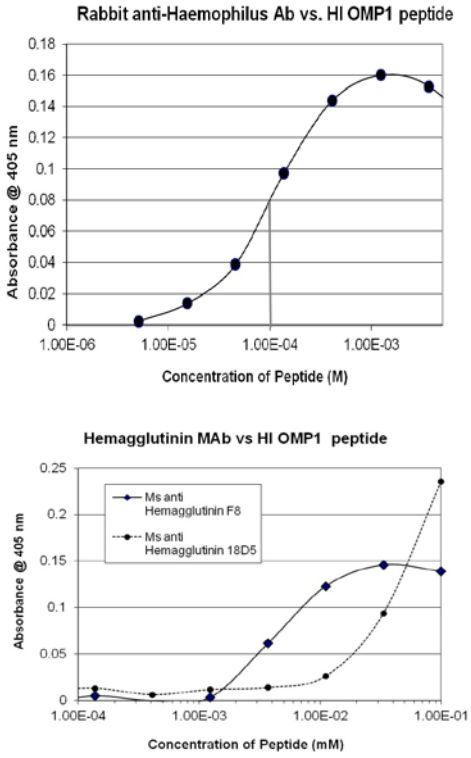

Figure 2: ELISA data showing that polyclonal antibody against HI recognizes the OMP 143-164 peptide used in these experiments as a dominant antigen; and that monoclonal antibody against IV H1N1 and H3N2 hemagglutinins recognize IV H3N2 306-318 as an antigen. It follows from Table 1 that this antibody against HI should recognize these antibodies against IV as "antigens" and vice-versa (see Table 5 and Figure 4).

Table 5: Complementarity of Haemagglutinin Antibody and H. Influenzae Antibody and Controls. Results of double antibody (DA)-ELISA experiments using antibodies against IV and adenovirus (left column) with antibodies against coxsackie virus B4, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. Binding constants (M) demonstrate that most of the antibody combinations yielded no measurable binding, but the combinations of IV with HI (10 to 50 nM) (Figure 4) and adenovirus with *S. pneumoniae* (5 μM) suggest that these pairs of infectious agents induce antibodies complementary to each other. Complementary antibodies would be induced by complementary antigens, as predicted by the data in Tables 1, 3 and 4 and Figures 1 and 2. The complementarity between *S. pneumoniae* and adenovirus appears to have a different molecular basis than that of IV with HI since no other antibody interactions between these agents occurred.

	Coxsackie B4	Staph aureus	Strep pneumoniae	H. influenzae
Influenza A H3N2	> 10^{-3}	> 10^{-3}	> 10^{-3}	**1 x 10^{-8}**
Influenza A H1N1	> 10^{-3}	> 10^{-3}	> 10^{-3}	**5 x 10^{-8}**
Influenza B	> 10^{-3}	> 10^{-3}	> 10^{-3}	> 10^{-3}
Adenovirus	> 10^{-3}	> 10^{-3}	**5 x 10^{-6}**	> 10^{-3}

Discussion

We used TCR sequences to identify proteins complementary to a specific haemagglutinin epitope of the IV thereby permitting us to identify sequences similar to the TCR that exist in infectious agents that synergize with IV. This technique of using TCR as clues to antigenic complementarity between diverse pathogens may have broader applications to exploring other pathogenic synergisms.

Our data demonstrate that a very highly conserved region of the hemagglutinin protein from type A influenza viruses binds with high affinity to a completely conserved region of the outer membrane protein of HI, which is also shared by a number of other respiratory bacteria that are often found as secondary infections to IV. Antibodies to IV recognize this hemagglutinin peptide as a major antigen, and antibodies to HI recognize this outer membrane peptide as a major antigen (Figures 1 and 2). Antibodies against IV and HI also recognize each other as antigens (i.e., they behave as idiotype-antiidiotype pairs, even though each is idiotypic) (Figures 3-5 and Table 5). Such antibody-antibody binding would result in neutralization of the antibody pair and drive increased antibody production as the immune system attempts to control a dual infection. We demonstrated that resulting antibody-antibody binding does interfere with binding to influenza viruses (in the form of vaccines), producing co-neutralization (Figures 6 and 7).

Similarity searching revealed a number of additional surprises related to the IV haemagglutinin and HI outer-membrane peptides used in these studies. These two peptides bind with very high affinity. The HI outer membrane protein therefore represents a potential binding site for type A IV. Notably, the HI outer membrane protein

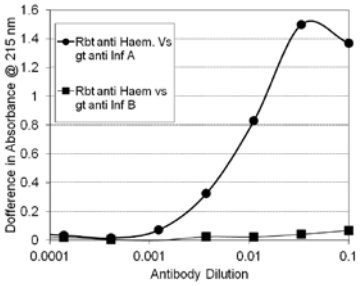

Figure 3: Results of a double-antibody (DA)-ELISA experiments showing that antibody against HI recognize as an antigen polyclonal antibody against IV type A but not IV type B. See Table 5 for additional negative controls.

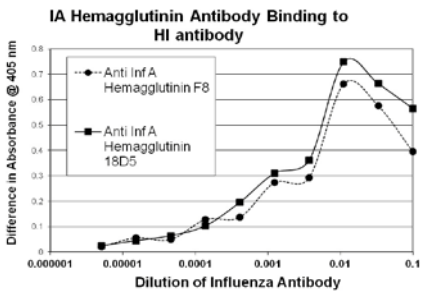

Figure 4: Double-antibody (DA)-ELISA experiments showing that antibody against HI recognizes as antigens monoclonal antibodies against H1N1 and H3N2 hemagglutinins as predicted by Table 1 and figures 1 and 2.

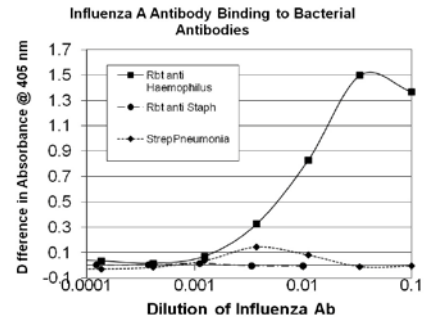

Figure 5: Double-antibody (DA)-ELISA experiments showing that polylonal IV antibody binds to HI antibody (see Figure 3) but not to antibodies against *S. aureus* or *S. pneumoniae* (see Table 5 for additional controls).

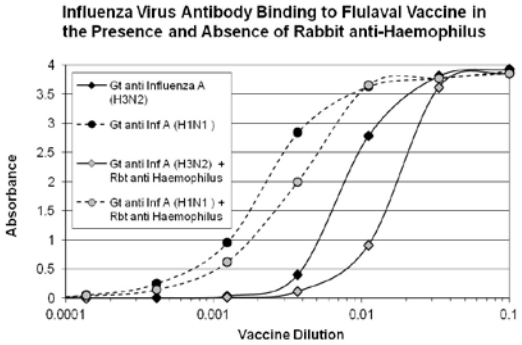

Figure 6: Competitive ELISA experiments showing that the presence of antibody against HI blocks polyclonal IV antibody binding to Flulaval 2007/2008 Vaccine (Glaxo Smith Kline) containing hemagglutinin from A/Solomon Islands/3/2006 (H1N1), A/Wisconsin, 67/2005 (H3N2), and B/Malaysia/2506/2004, as would be predicted from the data in Figures 3 and 5 and Table 5. Essentially identical data were obtained when and Fluogen Influenza A and B Vaccine 1987/1988 (Parke Davis) containing hemagglutinin from A/Taiwan/1/86 (H1N1), A/Leningrad/360/86 (H3N2), and B/Ann Arbor/1/86) was tested (data not shown).

is mimicked by several other proteins that may be of great importance to understanding lethal influenzas. In the first place, the HI OMP is mimicked by mucins (Table 6), which contain over 40 repeats of the HI OMP-like sequence. Just as we have demonstrated that IV binds to HI OMP, other investigators have demonstrated that IV binds to mucins [19,20]. We hypothesize that the binding sites for IV on mucins are the HI OMP-like regions. Notably, one of the functions of mucins is to prevent respiratory viral infection by binding up viruses [19,20].

HI OMP also mimics blood coagulation factor VIII (Table 6). We hypothesize that antibody against HI OMP protein may therefore cross-react with factor VIII resulting in acute autoimmune thrombocytopenia. Notably, coagulopathies were a very common symptom of the 1918-19 pandemic influenza and the 1928 hog epidemic [6,8]. These observations suggest that the complementarity of HI OMP to IV hemagglutinin reported here may provide important insights into the specific forms of morbidity associated with associated with their co-infection and rarely observed in their uncomplicated, individual infections.

We stress that other respiratory bacteria have outer membrane proteins that share a high degree of similarity with the HI OMP peptide studied here (Table 2). These include *K. pneumoniae, N. meningitides, A. pleuropneumoniae*, and *P. multocida,* all of which

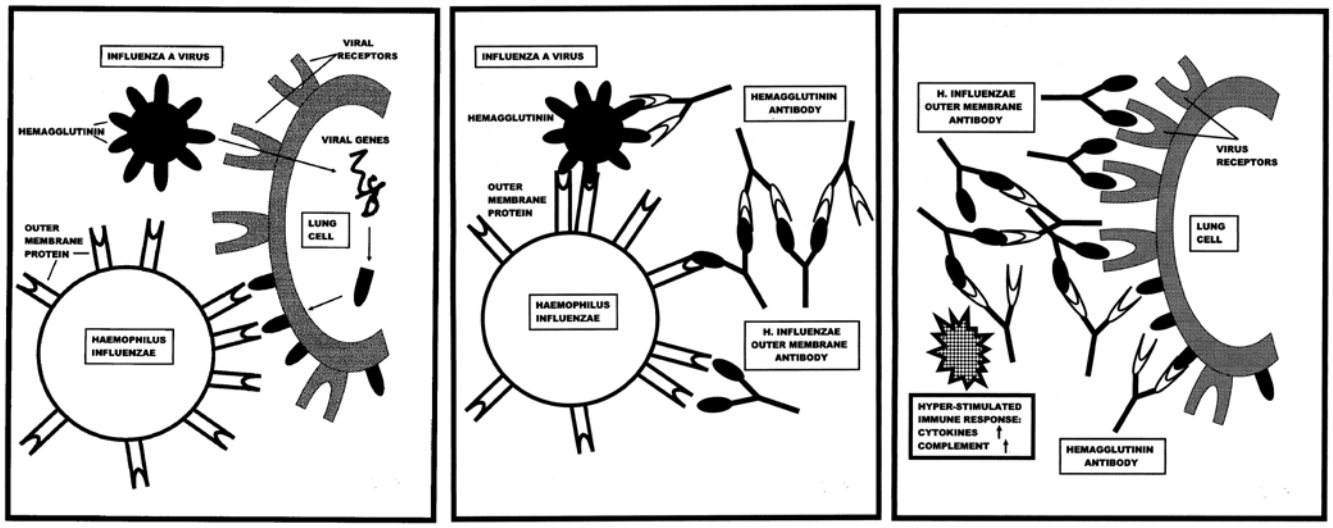

Figure 7: A graphic model explaining the data illustrated in Tables 1-5 and Figures 1-6. **A)** Influenza virus (IV) utilizes viral receptors to invade lung cells, which in turn express viral hemagglutinin on their cell surfaces. Outer membrane proteins (OMP) of H. influenzae (HI) are complementary to IV hemagglutinin and use the lung-cell expressed hemagglutinin to infect lung tissue. **B)** Complementarity between HI OMP and IV hemagglutinin has further implications. HI OMP can potentially bind directly to IV hemagglutinin so that the virus and bacteria "piggyback" on each other, increasing their dual infectivity. Meanwhile, HI OMP induced antibodies that mimic IV hemagglutinin and IV hemagglutinin induces antibodies that mimic HI OMP. These IV and HI antibodies recognize each other as antigens (i.e., they behave like idiotype-antiidiotype pairs). One consequence is that the antibodies against the infectious agents neutralize each other, becoming relatively ineffective against the infections. **C)** Once the "self-nonself" distinction is broken by the induction of pairs of idiotypic complementary antibodies, lung cells themselves may become targets of the antibody response, which would explain the devastating respiratory failure associated with combined infections. In addition, the combination of virus, bacterium, lung antigens, and antibody complementarity would result in ever-escalating antibody production accompanied by the kind of cytokine storm that is associated with rapidly fatal deaths in pandemic influenzas.

can cause bacterial superinfections of IV [21]. Thus, the possible mechanisms of immunological synergism found here, and developed below, may apply to a wider range of viral-bacterial co-infections such as those reportedly found in 1918-19 influenza victims by Morens et al. [15].

In order to understand the full implications of our results, it is necessary to consider them from several different perspectives concerning influenza virus and *Haemophilus* infections. To begin with, HI strains bind with varying affinity to mucosal, pharyngeal and lung tissues by means of various outer membrane proteins that recognize fibronectin, laminin, and collagen IV [20,22]. Pathogenic infection with HI, however, is much more likely following IV infection than in its absence [21], suggesting that additional IV-related factors increase colonization with HI. In this regard, Fainstein et al. [23], George et al. [24], and Hirano et al. [25] have demonstrated that influenza virus infection of pharyngeal and lung tissue results in the expression of increased numbers of binding sites for HI. While neuraminidase plays some role in facilitating bacterial co-infection following IV [26], much of the effect is produced by increased expression of hemagglutinin on the surface of infected cells [23,25,27]. The increases in HI binding are most dramatic for strains of HI that have the least natural affinity for the tissue [24].

One consequence of HI-IV co-infection should be a dramatic increase in the immune response to each infection (Figure 7C). Not only will each antigen stimulate its appropriate antibody, but each antibody will stimulate production of the other, at the same time neutralizing the antibodies' effects. This neutralization will have two concurrent results. One is that the immune response to both IV and HI will be impaired (Figures 5, 7B and 7C). This impairment will be further augmented by the fact that HI binds complement inhibitors such as Factor H and Factor H-like protein [28]. Colocalization of IV with HI by binding of IV to HI would interfere with anti-IV directed complement responses as well.

P0045 & A8KA37 cDNA FLJ77213		
P0045 Coagulation factor VIII 931-945	HYDSQLDTTLFGKKS	E 52
H. influenzae Outer Membrane P1, 143-163	SEYDDSYDAGIFGGKTDLSAI	
Q5HY69 Coagulation factor VIII 738-747	YEDSYE - - - - - - - - -DISA	E 40
P0045 & A8KA37 cDNA FLJ77213		
P0045 Coagulation factor 2144-2154	VFFGNVDSSGI	E 37
H. influenzae Outer Membrane P1, 143-163	SEYDDSYDAGI FGGKTDLSAI	
Q7Z5P9 Mucin-19 precursor 4834-4840	SAVVT- GKTELSA	E 48
H. influenzae Outer Membrane P1, 143-163	SEYDDSYDAGI FGGKTDLSAI	E 35
Q7Z5P9 Mucin-19 precursor 3570-3581	AGI - TGTTGLSA	
At least 40 repeats of these motifs within mucin		
Q9H792 Tyrosine-protein kinase SgK269, 308-314	DYDDSYD	E 52
H. influenzae Outer Membrane P1, 143-163	SEYDDSYDAG I FGG--KTDLSAI	
P06213 Insulin receptor 24-31 and 700-707	SEYEDS - -AGECCSCPKTD-SQ I	E 67

Table 6: Similarity of *H. Influenzae* OMP with Factor VIII and Mucins. A BLAST search of the SwissProt human database revealed that the HI OMP 143-163 sequence used in this study has a high degree of similarity to various human proteins, including blood coagulation factor VIII, the insulin receptor, and most especially with mucins. Mucins are involved with IV binding and clearance (19, 20), so antibody against HI OMP might interfere with mucin activity, increasing IV infectivity. Antibody against HI OMP might also block factor VIII, resulting in a form of autoimmune haemophilia, thereby explaining the uncontrolled bleeding associated with fatal cases of pandemic influenzae in 1918-19. And antibody against the insulin receptor could result not only in acute autoimmune diabetes, but in consequence, the significantly impaired T and B cell function that characterizes reduced resistance to infection among diabetic patients.

Secondly, antibody and/or T-cell co-neutralization may result in over-stimulation of the immune response. HI antibody (or T-cells) will stimulate IV antibody (or T cells) and vice versa, each recognizing the other as an antigen. Gönczi [29] has, in fact, reported that a combination of HI introduced into guinea pigs along with IV vaccine results in greatly increased antibody production against IV. In other words, HI acts as an "adjuvant" for IV. We predict that the converse is also true: that IV acts as an "adjuvant" for HI. The "co-adjuvant" effect may lead to over-stimulation of the immune response, or even a so-called "cytokine storm" such as that proposed to occur in pandemic influenzas [30]. It is also worth noting that a combination of IV with HI dramatically increases the incidence of meningitis in experimental animals [31]. Meningitis is also a common correlate of influenza pandemics.

Co-infection may not be limited during pandemic situations to coincidence of IV with HI in random patients. The complementarity of the IV hemagglutinin antigen and the HI OMP-P1 suggests that IV may be able to bind directly to HI, thereby "piggybacking" [32] on the bacterium (Figure 7B). Hament et al. [33] have shown that just such "piggybacking" occurs between respiratory syncytial virus and pneumococci. Avadhunula et al. [34] have demonstrated that HI and *S. pneumoniae* bind directly to a surface glycoprotein of respiratory syncytial virus, so that the probability of viral-bacterial co-infection is increased. The result of IV-HI piggybacking would be greatly increased invasiveness on the part of both virus and bacterium accompanied by the co-stimulatory immunological effects described above.

Co-infection and piggybacking of IV on HI could explain one of the great mysteries of the pandemic influenza of 1918-1919: its unusual mortality curve. Mortality due to IV in all recorded instances save the 1918-1919 pandemic resembles a "U" shape, in which the very young and the very old die, but people in their teenage through middle aged years are almost completely spared. The 1918-1919 influenza pandemic is exceptional in having a "W" shaped mortality curve. As in other influenza pandemics, the very young and the very old died but, oddly, those in their twenties and thirties were also at unusual risk of death [35,36]. One possible explanation of the "W"-shaped mortality curve is that pandemic influenza over-stimulates the immune system resulting in a "cytokine storm" that kills the patient, and the healthier the individual, the greater their likelihood of mounting such an over stimulated reaction [29]. This scenario could be explained in part by the mechanism proposed above in which complementary antibodies stimulate each others' over-production. We also propose an alternative possibility. Invasive HI infections are

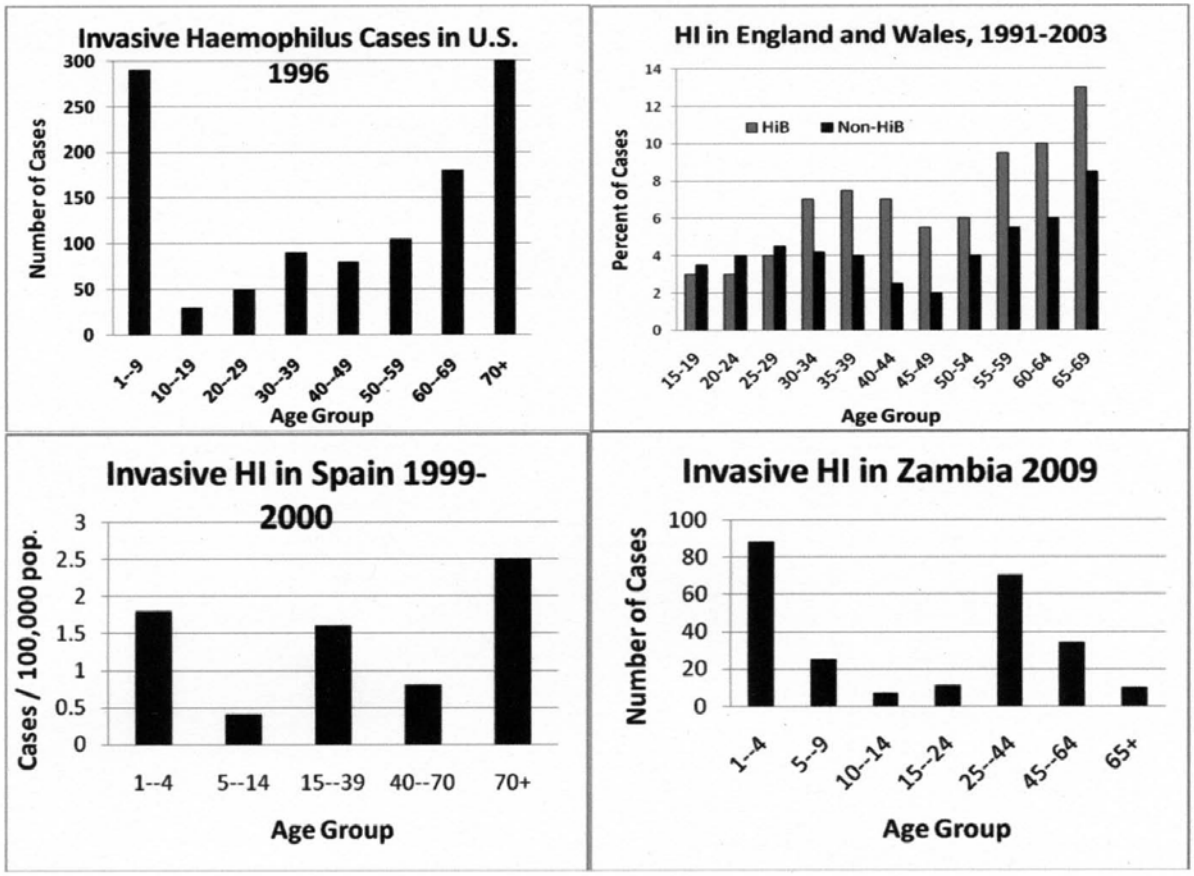

Figure 8: The 1918-19 influenza pandemic was uniquely characterized by a "W" shaped morbidity and mortality curve [35,36]. No other influenza virus outbreak has ever showed a similar "W" shaped curve, but the following data demonstrate that sometimes Haemophilus influenza (HI) cases display such a "W" shaped morbidity and mortality curve. Top left: Cases of HI in the United States in 1996, from [37]. Top right: Cases of HI in England and Wales, 1991-2003, from [38]. Bottom left: Cases of invasive HI in Spain, 2003, from [45]. Bottom right: Cases of invasive HI in Zambia, 2009, from [44]. Similar data showing a "W" shaped morbidity curve for HI can be found in references [39-43].

unusual among bacterial and viral infections in sometimes having a "W"-shaped incidence and case rate (Figure 8). Patients in their 20s and 30s contract HI infections at unusually high rates compared with those younger than 10 and those over 45 eg., [37-45]. Epidemiological evidence cited here is limited by the fact that only invasive disease and death caused by HI are reportable. Invasive HI may reflect the broader rates of non-invasive, non-lethal HI infections enlarging the W-shaped curves, or invasive HI may itself be a result of synergy with a virus such as IV therefore providing a natural model of pandemic influenza. In either case, we propose that HI synergizing with IV could have caused the "W"-shaped mortality curve that so unusually characterized the 1918-1919 pandemic. Infections other than HI might also synergize with IV to produce "W"-shaped mortality curves as well, but this possibility will require further exploration.

As just noted, of the risks just posed by conjoint HI-IV infections are also possible with other viral-bacterial respiratory co-infections. For example, Speshock et al. [46] have demonstrated that a combination of influenza virus type A with *S. pneumoniae* behaves just like Shope's combination of HI plus IV. Using a strain of IV that is non-lethal to mice, and a strain of *S. pneumoniae* that is non-lethal to mice, Speshock et al. [46] showed that the combination is rapidly lethal, producing an infection that disseminated widely to many organ systems and resulted in a hyper-immune response. HI has also been reported to synergize with respiratory syncytial virus, and IV with other bacteria such as *K. pneumoniae* [47], so that many other sets of co-infections may exist that work on the model proposed here and the immunological interactions of which might be elucidated through a similar approach.

Recognizing the potential hazards of IV-HI (and related) co-infections also suggests clear measures for interfering with their synergistic effects. While historians often scoff at reports of effective vaccines being developed against pandemic influenza during the 1918-1920 period, there are in fact a number of well-controlled, large-scale studies in which HI, often combined with Streptococcal strains, was used to infect or inoculate volunteers who were subsequently exposed to lethal cases of influenza [46-49]. While the methods and materials were not well controlled by modern standards, and efficacy varied, in several of these large-scale studies, unvaccinated volunteers died at significant rates while vaccinated volunteers developed fever and headaches, but no deaths occurred [48-52]. Such studies are worth taking seriously in light of Shope's subsequent experiments showing that pigs inoculated with either HI or IV were protected completely against the lethal effects of combined infection. The widespread introduction of Haemophilus type B (HiB) vaccine in the last two decades to prevent infant deaths may therefore have greatly lessened the risk of rapidly lethal outbreaks of viral influenza as well. The widespread use of *S. pneumonia* vaccines might have a similarly protective effect and if the 1919 experiments reported above are correct, a combination of such HI with Streptococcal vaccines might be a very effective prophylactic against future influenza pandemic deaths. In addition, the existence of broad-spectrum antibiotics, which were unknown in 1918, has changed the risk of death from bacterial co-infections of IV significantly. All of these factors suggest that highly lethal pandemics of influenza of the past may be largely preventable.

In sum, we suggest that the 1918-19 pandemic was really *two concurrent, synergistic pandemics*, and that co-pandemics of viral influenzas with bacterial "influenzas" may be of more concern than pandemic influenza alone. The challenges posed by pre-existing combined viral-bacterial infections is that they are often resistant to antibiotic treatment [47] and do not respond well to antivirals such as oseltamivir, either [53]. In consequence, while stockpiling antibiotics for use in an influenza pandemic is an important precaution [14], antibiotics cannot be relied upon to treat combined infections, particularly in immunocompromised and malnourished people. We propose that the objective of public health policies must be to prevent the co-incidence and co-transmission of such mixed infections in the future. One approach to prevention would promote widespread prophylactic use of Hib, streptococcal with other respiratory bacterial vaccines, particularly among young adults. Hib and streptococcal vaccines in combination have been reported to halve the incidence of radiologically-diagnosed pneumonias [54,55] in children and might have a similar protective effect in adults. Additionally, the development and stockpiling of such bacterial vaccines is likely to be simpler than the current strategy of attempting to develop a novel IV vaccine at short notice once an epidemic has begun. We note, however, that no vaccine yet exists against non-B HI of some of the other bacterial infections for which we present the possibility of IV synergy.

One final caution is necessary. The data presented here strongly argue against the untested, conjoint use of respiratory bacterial vaccines at the same time as, or mixed with, influenza virus vaccines. In the absence of antigen deletion of complementary proteins, combining such vaccines may be as dangerous as contracting the corresponding pair of infections and might lead to increased risk of meningitis [31]. The co-adjuvant effects of HI and IV antigens [29] might, alternatively, be harnessed to produce much more effective vaccines that protect vaccinated individuals against both infections. The risk-benefit ratio of such antigenic interactions can only be understood through further research.

In summary, we cannot assume that highly lethal pandemic influenza is merely an unusual genetic variant of normal strains of IV [11], though some strains of IV are certainly more easily transmissible and more pathogenic than others; nor can we discount the historical record of the presence of HI in human and porcine "influenza" epidemics [1-10]. New studies of the interactions between viral and bacterial respiratory disease agents (not limited to HI), their combined effects on immune function, and more effective ways to treat such combined infections are needed. It is possible that vaccination against bacterial co-infections of IV may be more beneficial and easier to accomplish than rapid development of vaccines against new IV strains and therefore of more utility in preparing for, or preventing, future pandemics. It is also be possible that only certain strains of influenza viruses can synergize antigenically with particular strains of Haemophilus. Studies of the determinants of synergism might be of great benefit in monitoring the risk of lethality in future pandemics as well as for understanding the pneumonias and meningitis that afflict some influenza patients during every influenza virus outbreak. In sum, disease synergism may drive the lethality of some pandemics, in which case single-agent approach to prevention, treatment, and epidemiological tracking may not suffice.

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