Internal Medicine Grand Rounds
May 10, 2001

Influenza: New insights, new therapies

James P. Luby, M.D.
Professor

Division of Infectious Diseases

*"This is to acknowledge that James P. Luby, M.D., **has no** financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Luby will not be discussing "off-label" uses in his presentation."*

Introduction

This discussion will concentrate on influenza A virus but will also include influenza B virus. Advances in modern virological techniques have advanced our understanding of influenza A virus and the way that it has evolved. There is now substantial evidence that influenza A is a zoonosis and that pandemic strains either arise from genetic reassortment between an avian influenza A virus and a human influenza A virus or else that an avian influenza A virus directly becomes adapted to humans without an intermediate step. For example, an average amino acid number in an hemagglutinin monomer approximates 570. There are only 16 amino acid differences between HA2 and its closest avian counterpart. With respect to HA3, there are only 10 amino acid differences. HA5 contains the same amino acids with its avian counterpart except for 3 differences. In a remarkable series of ongoing investigations, the 1918 influenza A virus has been resurrected. Influenza A RNA sequences were obtained from the paraffinized lung of a soldier in South Carolina dying in 1918 from influenza A. Influenza A RNA segments were also been obtained from a soldier who died in 1918 in New York. The lung of an Eskimo woman buried in the permafrost in Alaska also contained influenza A RNA. Of note, the RNA segments from each of these patients was never longer than 120 nucleotides. It has been possible by RT-PCR and PCR to sequence the hemagglutinin and the neuraminidase of the 1918 virus as well as its nonstructural proteins, NS and NS1. These sequences in their cDNA configuration were then compared with sequences from multiple other H1N1 viruses and from the swine influenza (HswN1) virus that Dr. Richard Shope isolated from pigs in 1931. It has been possible to place the 1918 viral segments in phylogenetic trees and to ascertain their relationships with these other

viruses. It has also been possible to search for mutations which confer virulence by examining the sequences of these proteins. The nonstructural proteins of the 1918 influenza A virus have been sequenced and these proteins have been placed back into a reassortant virus whose other genomic segments were derived from the WSN variant of the H1N1 virus. A cold adapted (ca) live attenuated virus vaccine has been tested successfully in children and should be ready for marketing within the next year. Progress has been made in the rapid detection and identification of influenza virus from the human nasopharynx and/or pulmonary secretions so that the diagnosis can be accomplished within hours of receipt of the specimen in the laboratory. Rapid diagnosis should aid in the way we manage patients in terms of therapy but also because hospitalized persons can be isolated early in their course. The structure of the neuraminidase active site has been elucidated and molecules have been designed to interfere with the action of the active site on receptor sialic acid residues. These antiviral drugs, the neuraminidase inhibitors, have been produced and are now available for use in both children and adults to complement the M2 protein inhibitor drugs (amantadine, rimantadine) for the therapy of influenza.

Basic Virology

Influenza A differs from influenza B virus in that the ribonucleoprotein of each appears to be antigenically distinct. Influenza C virus also has a distinct ribonucleoprotein. Influenza A is an enveloped RNA virus, which is usually spherical and has 3 projections from the surface envelope. The surface projecting proteins are the hemagglutinin, the neuraminidase and the M2 protein, which acts a hydrogen ion channel. Under the envelope is matrix protein and underneath that protein and attached to it are 8 ribonucleoprotein coated genomic segments coding for 10 proteins. There is an

attachment of the matrix protein with ribonucleoprotein, which must be lysed before the genomic segments can make their way into the cytoplasm of the cell. Each genomic segment is wound as a heliconucleocapsid and then turned back upon itself to form a panhandle structure. The 5^1 and 3^1 ends are connected to each other. Each genomic segment contains one or more transcriptase complexes. A transcriptase complex consists of a polymerase protein that is basic (PB1) and another basic polymerase protein (PB2). There is an acidic protein, which also functions as a polymerase, called PA. The 8 genomic segments code for HA, NA, RNP, PB1, PB2, and PA; the matrix proteins M1 and M2 are coded by 1 genomic segment and another segment codes for 2 nonstructural viruses, NS1 and the nuclear export protein (NEP). The hemagglutinin monomer is approximately 570 amino acids long; it is divided into 2 parts, HA1, which attaches to the sialic acid receptor sites on the epithelial cell surface and HA2, which acts as the fusion protein and is joined to HA1 by disulfide linkages. The hemagglutinin is a trimeric protein. The attachment site is at the end of HA1 at the terminal portion of the hemagglutinin. It is hidden from antibody molecules by glycosylation sites. There are several important antigenic regions around the receptor site, which mutate with time producing antigenic drift. The hemagglutinin attaches to the receptor on the cell surface in an area where there is a clathrin-coated pit. Endocytosis ensues and the endosome fuses with a lysosome. In the presence of intracellular or extracellular proteases, the hemagglutinin is cleaved to expose HA2, which then acts to fuse the envelope of the influenza virion with the envelope of the endolysosome. When single or multiple basic acids are placed at the cleavage site, HA2 is exposed more easily and such mutations confer virulence to the virus. After fusion has occurred, the ribonucleoprotein complexes

must be freed from their attachment to the overlying matrix protein. This is accomplished by the passage of hydrogen ions through a hydrogen channel. In the presence of a functioning M2 protein, the ribonucleoprotein segments are detached from the M protein and make their way into the cytoplasm. Amantadine and rimantadine interfere with the function of the hydrogen ion channel and are known as M2 protein inhibitors. The transcriptase complex along with the ribonucleoprotein direct the entrance of each of the genomic segments into the nucleus through a nuclear pore. The transcriptase complex then makes messenger RNA (mRNA) and a template for viral replication (template RNA), which can be transcribed back to viral RNA (vRNA). The transcriptase complex produces a polymethylated cap at the 5^1 end of the virion, a segment of invariant nucleotides, which are followed by messenger RNA and a polyadenylated tail. The messenger RNA is exported back into the cytoplasm where it binds to ribosomes and induces protein translation. Specific proteins are further processed by glycosylation at the Golgi apparatus. Both the hemagglutinin and the neuraminidase are glycosylated. After glycosylation, these proteins move to the cell surface along with the M2 protein. The matrix protein underlies the plasma membrane containing HA, NA and M2. The process of packaging occurs which may be inefficient in that after a replicative cycle only about 10% of virions are infectious. An infectious virion may contain 8 or even more genomic segments. It is, however, essential that all 8 genomic segments be contained within a single particle. The virus then buds from the cell, remaining attached through sialic acid residues, which are cleaved enzymatically by the neuraminidase. Virions have a tendency to retain some sialic acid residues on their surfaces and autoaggregation may occur which is prevented by the neuraminidase.

Finally, there is attachment of the virion to proteins and mucus and the neuraminidase can lyse that attachment allowing the virion to remain free in respiratory secretions. The neuraminidase inhibitors, zanamivir and oseltamivir, are compounds that are designed to affect the activity of the neuraminidase function and they have been proven to act beneficially in human infections.

Genetic reassortment occurs when two different influenza A viruses infect a single cell and the progeny viruses contain genomic segments from each of the parents. It has recently been shown that live influenza A viruses can be constructed by use of a 12 plasmid system and either Vero or human embryonic kidney cells. Eight of the plasmids are cDNA copies of the original genomic segment preceded by an RNA polymerase promoter and followed by an appropriate termination sequence. The remaining 4 elements are protein expression plasmids, which initiate the direct production of the transcriptase complex proteins (PB1, PB2, PA, RNP) after the plasmids are transfected into the cell. In such a system (genomic segment linked with tranacriptase complex proteins), live virions capable of independent replication can result. This process paves the way for genetic studies because parts of different genomic segments can be placed into the background of a single virion. For example, it has been found possible to place the NS genomic segment and the NS1 gene of the 1918 virus into a viable influenza A virion with the 7 other genomic segments being supplied by a H1N1 WSN virus. By such reverse genetics, it should be possible to produce a viable 1918 virus. The influenza A virion is constantly drifting, that is, changing its nucleotide and amino acid structure in response to the pressure placed upon it by immune mechanisms.

Influenza A as a Zoonosis

Although it has been widely know for a long time that influenza A viruses circulate in non-human animals, the critical importance of these viruses has only recently began to be realized. Webster and Laever made the observation that the human HA3, which began to circulate in 1968, had an amino acid sequence that was just 10 amino acids different from that of a circulating avian influenza HA3 virus. Now, it has been determined that there are 15 hemagglutinin subtypes each of which differ by 30% from the others that currently exist in the world. There are 9 neuraminidase types that currently exist in the world. This makes for a potential total of 135 hemagglutinin and neuraminidase combination possibilities. These combinations exist and are propagated in a relatively unchanging state for the most part in avian species, particularly in waterfowl like ducks. Influenza A viruses also presently circulate in pigs and horses and have infected whales, sea lions and other animals. For aquatic avian species there appears to be very little immune pressure for evolutionary changes in the hemagglutinin or the neuraminidase. All of these HAs and NAs presently persist in nature relatively unchanged. In 1957, there was a genetic reassortment event in which an H1N1 virus reassorted with an avian virus containing an H2N2 hemagglutinin and neuraminidase. The PB1 from the avian virus also was inserted into the new reassortant. Thus, the 1957 H2N2 virus consist of 3 genetic segments derived from an avian species and 5 genetic segments derived from an H1N1 virus. The proof of this postulate was conformed by direct sequencing of each gene segment. The H2 human hemagglutinin differed by 16 amino acids from its closest avian counterpart. In 1968-1969, another pandemic exchange occurred wherein avian H3 and an avian PB1 reassorted with the H2N2 virus.

The new H3N2 virus that resulted had 2 genetic segments derived from an avian virus and the 6 remaining ones from the H2N2 virus. These conclusions were drawn from comparisons of the sequences of each of the genomic segments of the new H3N2 virus.

In 1997, in Hong Kong an unusual even occurred. There were hundreds of thousands of cases of influenza in avian species in and around Hong Kong and South China that year. An H5N1 virus was isolated from humans; all of its 8 genomic segments were derived from a bird influenza virus. The H5N1 virus infected 18 humans of whom 6 died, for a case fatality rate of 33%. On the basis of serological evidence of infection, persons caring for the cases also were involved. The epidemic in humans was aborted by the slaughter of over a million birds. In 1999, an H9N2 virus emerged and infected two children. The H9N2 virus contained surface proteins endogenous to avian species around Hong Kong and South China and the 6 other genomic segments had identical sequences to the H5N1 virus. The H5N1 virus was virulent because of the placement of basic amino acids at the HA cleavage site. It is conceived that Southern China and the area around Hong Kong represent a fertile area in which an interchange of human avian and other mammalian viruses may occur and this region may be an epicenter for the creation of pandemic influenza A viruses.

Resurrection of the 1918 Virus

In 1889-1890, an influenza pandemic struck the world. In testing the sera of persons who lived through that period and which were collected before the 1957 and 1968 pandemics, it has been established that the pandemic most likely was caused by an H3N8 virus. Although that population from whom the serum samples was taken does have some H2N2 antibody, that antibody was not considered of high enough magnitude

nor in a sufficient number of people to conclude that an H2N2 virus also circulated in the latter part of the nineteenth century. Presumably, this H3N8 virus circulated until or shortly before the great pandemic occurred. This pandemic caused the lives of at least 25 million people around the world and was remarkable in causing the deaths of a disproportionate number of persons infected in the 15-44 year old age group. The first historical occurrence of the pandemic occurred in the spring of 1918, at Camp Funston in Fort Riley, Kansas. The virus was highly infectious, induced immunity but was not lethal. In September and October 1918 simultaneously in many parts of the world, the great pandemic occurred with the highly lethal virus and persisted into the spring of 1919.

Historically, disease resembling influenza appeared in swine during the autumn 1918 epidemic. In 1931, Richard Shope isolated the swine influenza virus (HswN1) and showed that persons living through the 1918-1919 epidemic had neutralizing antibodies against it. In 1933, Smith, Laidlaw and Andrews isolated the first human virus in ferrets. It was labeled as an H0N1 virus to distinguish it from HswN1, which caused the great pandemic. In 1929, there was sufficient numbers of cases that a new pandemic was thought to have occurred producing the new H0N1 virus. In 1947, another widespread epidemic occurred sufficient to suggest a new pandemic virus strain namely H1N1. It is now apparent from sequence analysis, that all of the viruses circulating from 1918 to 1957 were of the H1N1 subtype. The 1929 and 1947 epidemics resulted from the process of antigenic drift, which then generated large numbers of cases.

The mystery that has plagued man since the 1918 pandemic is what was the nature of the virus, where did it originate and what was the basis of its remarkable

virulence. Young men were noted to die within a day or several days after being infected, usually of hemorrhagic pneumonia. As a result of modern virological techniques, it has been possible to resurrect the 1918 virus. Using paraffinized sections of lung obtained from 2 soldiers and frozen lung from an Inuit woman infected during the 1918 epidemic, 3 isolates have been obtained and are in the process of being sequenced. One isolate was from the lung of a soldier dying in New York with influenza pneumonia, another isolate was from a soldier in South Carolina and the third isolate was from the frozen lung of a woman dying with hemorrhagic pneumonia in Alaska. In the Alaskan village where this Eskimo woman died (Brevig Mission) there were 72 adult deaths, 85% of the village's adult population. Using RT-PCR techniques and PCR, it has been possible to reconstruct the 1918 virus and there are now articles describing the sequences of the 1918 hemagglutinin, neuraminidase and the NS and NS1 gene segments of the virus. It is expected that the sequences of the entire virus will be elucidated within the next 2-3 years. Sequence analyses of these 3 gene segments place the virus into the H1N1 subtype. The 1918 virus is most closely related to HswN1 isolated from pigs in 1931. The 1918 virus, however, has many avian features. For the hemagglutinin, in one of the patients there was a single amino acid change in its attachment site. In the other two patient isolates, there were two amino acid changes in the attachment site compared to the closest H1N1 avian virus. Of the 41 amino acids in the 1918 virus subject to intense immune pressure in humans, 37 match the avian consensus sequence. Modern human H1N1 HAs have up to 5 glycosylation sites in addition to the 4 found in all avian strains. The 1918 virus has only the 4 conserved avian sites. Basic amino acid substitutions around the cleavage site were not noted to occur in the 1918 virus. These

were noted in the H5 avian virus isolated in 1997 and eliminates that possibility as a potential virulence factor for the 1918 virus. The neuraminidase active enzymatic site is identical between H1N1 avian viruses; it was shared by the 1918 virus. Of the neuraminidase sites subject to antigenic drift and common to N1 and N2, 21/22 match the avian consensus sequence. The 1918 NA has identical glycosylation sites to avian H1N1 strains. Loss of a glycosolation site in the neuraminidase renders the H1N1 virion more virulent and capable of attacking the central nervous system; this was not found in the 1918 virus. The NS gene codes for the nonstructural protein 1 (NS1) which is a type 1 (alpha) interferon inhibitor. It also codes for the nuclear export protein (NEP). Phylogenetic analyses of NS and NS1 place the 1918 virus within or near the root of the human/swine clade of viruses. Under BSL 3+ antigen containment, transfectant viruses were generated containing 7 genomic segments from the mouse adapted WSN H1N1 virus and either the 1918 NS1 ORF alone or the entire 1918 NS segment. The 1918 transfectant viruses replicated well in tissue culture but were attenuated in mice compared with isogenic control viruses.

The best hypothesis concerning the origin of the 1918 virus from phylogenetic trees and regression lines derived from amino acid changes from antigenetic drift is that this virus probably arose from an avian progenitor sometime between 1915 and 1918, became adapted to humans and most probably then spread to swine. It seems most likely that all of its genetic information was of avian origin but this remains to be proven and as yet there is no explanation for its hypervirulence. Planned future studies will attempt to reconstruct the 1918 virus protein by protein by reverse genetics using the 12 plasmid strategy outlined previously. The mechanism(s) of its hypervirulence should be able to

be ascertained and this will be new knowledge that may make dealing with pandemic viruses less difficult. It may be related to a single gene or the interaction of several genes and obviously must be accomplished under the highest possible safety containment. The editorial accompanying the generation of the 1918 NS segment transfectant calls attention to the potential consequences of the use of this technology for destructive purposes and of the necessity to be certain that this does actually happen.

In 1976, the swine flu incident occurred. During the spring 1976 in Fort Dix, New Jersey, swine influenza virus was introduced into the recruit population of Fort Dix. An epidemic ensued which caused over one hundred cases and one death. It was thought that an epidemic of this magnitude most likely was caused by a virus (A/New Jersey/8/76) that was a reassortant between HswN1 and H3N2. Since it was antigenically distinct, it was thought capable of pandemic spread and displacing the H3N2 virus. Human experience with HswN1 was thought to be limited (1918-1929). It was not realized that HswN1 was a H1N1 subtype and that the population had extensive (1918-1957) experience with this group of viruses. The incident resulted in the production of a vaccine that was eventually administered widely to millions of persons. The vaccine campaign was aborted because there were no further cases caused by this virus and because the vaccine was associated with a higher rate of the Guillan-Barre' syndrome. In actuality, this virus was not a reassortant but merely a swine influenza virus that was simply easily transmitted in military recruit circumstances and which subsequently disappeared. In 1977, an H1N1 virus almost identical to one isolated in 1950 emerged. It occurred in the setting of the continuing transmission of an H3N2 virus. It did not displace the H3N2 virus and now circulates with it. The 1977 H1N1 virus is considered

to be of Eurasian origin, from either Russia or North China. Since this virus is so similar to a 1950 virus from humans and phylogenetically distant from avian and swine H1N1 viruses, it has been suggested that it arose from "cold storage" and actually may have escaped from a laboratory. There is no direct proof that this occurred and its origin remains an enigma.

Influenza Vaccines

The presently available inactivated influenza vaccine is usually a split virus vaccine, that is it contains a purified hemagglutinin and neuraminidase from an H3N2 virus, an H1N1 virus and a B virus. The antigenic determinates included in the vaccine preparation are subject to intensive discussions by experts throughout the world. Important in the determination of the constitution of the vaccine is ascertaining which virus most successfully is transmitted late in the influenza season and which virus is transmitted most widely in the southern hemisphere during our summer. Because of laboratory capability, the southern hemisphere viruses proposed for vaccine consideration are usually from Australia or South Africa. In adults, only one vaccine dose is necessary and is usually given before mid-October and produces immunity through the influenza season, which generally extends through March. A meta-analysis of multiple studies has been done comparing the efficacy of vaccine to placebo in elderly persons. When the vaccine strain is similar to the strain that later circulates, almost all the studies demonstrate a significant benefit. An overall protective efficacy approximates 65% and this extends to laboratory proven cases of influenza, pneumonia and mortality. Influenza vaccines are inexpensive, they are widely available and when given yearly the protective efficacy may increase although this is debated. They are not without complications and

an occasional case of the Guillan-Barre' syndrome may occur after administration but this has been a rare event since the 1976 swine flu incident.

A new influenza vaccine has become available utilizing a cold adapted (ca) virus developed by Masaab and associates. Using genetic reassortment, it is possible to take a parent strain that is cold adapted and avirulent and infect cells with this virus and the virus with the surface proteins most likely to be transmitted during the course of the ensuing influenza season. After selection a vaccine virus is produced that is not capable of reversion back to wild type and is poorly transmissible but has the surface proteins of the circulating virus. The vaccine can be given by nasal drops and is much easier to administer to children. The efficacy of one dose of the ca vaccine is almost equal to that of two dosages. In children, usually it takes at least two injections of the inactivated vaccine in order to be protected. These ca vaccine viruses can cause rhinorrhea and a sore throat but with these exceptions, complications are usually not different from placebo. In a recent study, vaccine administration led to a reduction of 30% of cases of febrile otitis media in children. These vaccines have been approved for use by the FDA but in the year following the publication of the study demonstrating their efficacy, inspection of the manufacturing process found inadequacies. It is expected that this vaccine will be approved for distribution in 2001-2002. This new live virus vaccine does not offer a sufficient advantage in adult populations for it to be preferred over inactivated vaccine. It is possible that the combined use of the inactivated vaccine in adults and the live ca vaccine in children will be a subsequent immunization strategy for prevention of influenza morbidity.

Influenza Diagnostic Tests

Influenza viruses, both A and B, are easily grown in the laboratory in tissue culture systems and their identity can be ascertained readily by staining infected cells with monoclonal antibodies tagged with flourescein. Tissue culture remains the 100% standard but lately there have been attempts at methods that would produce more rapid identification of the viruses using nasopharyngeal washes or from nasopharyngeal swabs. Cells can then be isolated, washed and stained by indirect immunofluorescence using monoclonal antibodies to ascertain the presence of virus in respiratory epithelium. This is a rapid process completed within two hours after receipt of the virus in the laboratory and is consistently sensitive and specific. Critical to the success of the procedure is to obtain specimens of the highest caliber. There are other rapid methods available. An EIA method detects viruses by fixing antigen to a membrane and subsequently adding antibody tagged with an enzyme. If the virus is present a color change is observed upon addition of a chromogenic substrate. Another method relies upon detection of neuraminidase in respiratory secretions on the basis of a chemical reaction and does not differentiate between influenza A and B viruses. If neuraminidase is present in secretions it can be processed so as to act enzymatically on a substrate to release a colored compound enabling the detection of either influenza A or B virus. The use of rapid influenza detection tests should be helpful in patient care and elucidating which patients have influenza when they are admitted into the hospital so that they can be properly isolated. Ascertaining whether a patient has influenza A or B should be important in determining which antiviral agent may be best for therapy when the illness is recognized early. Although these latter tests should become widely available it is probably optimal

for quality assurance purposes to perform them at some facility in which tests are done daily along with immunofluorescence and where viral cultures are also performed. In metropolitan areas, viruses also need to be typed (H1N1, H3N2) and a certain number of isolates need to be sent to the State Health Department to see if there has been any significant antigenic drift in isolates in the community during the course of the year.

Therapy of Influenza Infections

There are now four drugs that have been approved by the FDA for the treatment and prevention of influenza virus infections. Amantadine and rimantadine are useful for influenza A virus infections if given within 48 hours after onset. They inhibit the hydrogen ion channel or the M2 protein so that hydrogen ions cannot be transported into the virion from the endolysome. The ribonucleoprotein, therefore, cannot be detached from the overlying matrix protein. These drugs prevent entry of the genomic segment into the cytoplasm. Mutations in the M2 protein are associated with resistance to both amantadine and rimantadine. Resistance mutations have been found to occur not infrequently in a setting where patients are treated with amantadine or rimantadine and close relatives are also being given one of these drugs for prophylaxis. The development of resistance may limit the widespread use of these two drugs. Amantadine is not metabolized in the body but is excreted into the urine as an intact molecule. Its dose must be reduced with renal dysfunction. It has central nervous system side effects such as jitteriness and insomnia. The ordinary adult dose is 100 mg twice a day. In persons over the age of 65, the therapeutic dose is 100 mg per day. Therapy usually is given for 5 days. In one study, amantadine when given within 48 hours of the onset of illness, made a subset of patients into rapid responders, that is they responded to the drug rapidly and,

within 1-2 days were well. Rimantadine is excreted into the urine but it is also metabolized so that it has a shorter half-life than amantadine. Its dosage is identical to amantadine but is reduced less with renal dysfunction. Usually, studies find the duration of illness with both drugs to be shortened by at least 1-2 days. There is no evidence that complications after influenza are prevented by therapy with these drugs and no studies documenting an effect in treating established influenza pneumonia. Both drugs are inexpensive. Rimantadine is usually preferred because of its relative lack of central nervous system side effects.

Two new drugs have been FDA approved for the treatment of both influenza A and B. They are zanamivir and oseltamivir. The drugs were developed by examining the structure of the active site of neuraminidase and designing molecules, which would interfere with the activity of that enzyme on sialic acid residues on the cell surface. Since both influenza A and B viruses have a neuraminidase they can both be treated with either zanamivir or oseltamivir. Zanamivir comes as an inhaled powder and the patient must use an inhaler for delivery of the 10 mg dose used for therapy twice a day for 5 days. Prophylaxis requires 1 dose per day. When given to people with reactive airways disease, zanamivir can induce worsening of airway obstruction. Oseltamivir can be given orally for therapy at a usual adult dosage of 75 mg twice a day for 5 days. Prophylaxis requires 1 dose per day. Both drugs must be given within 48 hours of the onset of illness. Both drugs shorten the duration of illness by at least 1-2 days and complications of influenza may be prevented. With prophylaxis both drugs have an efficacy approximately 65% and they can be given for as long as 6 weeks. There is no evidence of their efficacy in established influenza pneumonia. With oseltamivir there are very few

side effects aside from headache, nausea and vomiting in a few patients. The cost of the neuraminidase inhibitors is comparable but more than the H2 protein inhibitors. If only influenza A is present in the community, the best practice would be to give either amantadine or rimantadine for influenza illnesses on the basis of cost-benefit considerations. If both influenza A and B are in the community at the same time, then a neuraminidase inhibiter probably should be utilized as the drug of first choice if a specific diagnosis cannot be made. If a specific diagnosis can be made, therapy can be directed appropriately. Such a rapid diagnosis made be able to be made by the newer influenza antigen detection tests with or without the use of immunofluorescent techniques.

Basler CF, R. A., Dybing JK, Janczewski, TA, Fanning, TG, Zheng H, Salvatore M, Perdue, ML, Swayne DE, Garcia-Sastre A, Palese P, Taubenberger JK (2001). "Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes." Proc Natl Acad Sci USA **98**(5): 2746-2751.

Belshe RB, B. B., Newman F, Cerruti RL, Sim IS (1989). "Resistance of influenza A virus to amantadine and rimantadine: results of one decade of surveillance." J Infect Dis **159**: 430-35.

Bot A, S. M., Bot S, Woods C, Limmer J, Kennedy R, et al (1999). "Induction of antibody response by DNA immunization of newborn baboons against influenza virus." Viral Immunology **12**(2): 91-96.

Bucher DJ, K. I., Lvov DK, Pysina TV, Lee HM (1980). "Comparative study of influenza virus H2 (Asian) hemagglutinins isolated from human and avian sources." Intervirology **14**(2): 69-77.

Bush RM, B. C., Subbarao K, Cox NJ, Fitch WM (1999). "Predicting the evolution of human influenza A." Science **286**(5446): 1921-5.

Chen Z, K. S., Hagiwara Y, Yoshikawa T, Matsuo K, Kurata T, Tamura S (2000). "Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase." Vaccine **18**(28): 3214-22.

Clements ML, B. R., Murphy BR. (1984). "Advantage of live attenuated cold-adapted influenza A virus over inactivated vaccine for A/Washington/80(H3N2) wild-type virus infection." Lancet **1**: 705-708.

Couch, R. B. (1993). "Advances in influenza virus vaccine research." Ann N Y Acad Sci **685**: 803-812.

Couch, R. B. (2000). "Influenza: Prospects for Control." Annals of Internal Medicine **133**(12): 992-998.

DA, S. (1999). "Role of hemagglutinin cleavage for the pathogenicity of influenza virus." Virology **258**(1): 1-20.

de Bruijn IA, R. E., Jol-van der Zijde CM, van Tol MJD, Westendorp RGJ, Knook DL (1999). "Qualtiy andquantity of the humoral immune response in healthy elderly and young subjects after annually repeated influenza vaccination." J Infect Dis **179**: 31-36.

de Bruijn IA, R. E., Jol-van der Zijde CM, van Tol MJD, Westendorp RGJ, Knook DL (1999). "Quality and Quantity of the Humoral Immune Response in Healthy Elderly and Young Subjects after Annually Repeated Influenza Vaccination." The Journal of Infectious Diseases **179**: 31-36.

de Jong JC, B. W., Palache AM, Rimmelzwaan GF, Osterhaus AD (2000). "Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly." J Med Virol **61**(1): 94-9.

Deguchi Y, T. Y., Tatara K (2000). "Efficacy of influenza vaccine in the elderly in welfare nursing homes: reduction in risks of mortality and morbidity during an influenza A

inhibitor oseltamivir to prevent influenza." NEJM **341**(18): 1336-43.

Hayden FG, B. R., Clover RD, Hay AJ, Oakes MG, Soo W (1989). "Emergence and apparent transmission of rimantadine-resistant influenza A virus in families." N Engl J Med **321**: 1696-1702.

Hayden FG, O. A., Treanor JJ, et al. (1997). "Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza virus infections." N Engl J Med **337**: 874-880.

Hayden FG, T. J., Betts RF, Lobo M, Esinhart JD, Hussey EK (1996). "Safety and efficacy of the neuraminidase inhibitor GG167 in experimental human influenza." JAMA **275**: 295-99.

Hurwitz ES, H. M., Chang A, Shope T, Teo ST, Giesick JS, Ginsberg MM, Cox NJ (2000). "Studies of the 1996-1997 inactivated influenza vaccine among children attending day care: Immunologic response, protection against infection, and clinical effectiveness." J of Infect Dis **182**: 1218-1221.

Jefferson TO, D. V., Deeks JJ, Rivetti D (2001). "Amantadine and rimantadine for preventing and treating influenza A in adults (Cochrance Review)." The Cochrane Libary(1).

Joshua, L. (2001). "H1N1-influenza as Lazarus: Genomic resurrection from the tomb of an unknown." Proc Natl Acad Sci USA **98**(5): 2115-2116.

KF, S. (1992). "Pandemic Influenza: A Zoonosis?" Semin Respir Infect **7**(1): 11-25.

KF., S. (1995). "The next pandemic influenza virus?" Lancet **346**: 1210-1212.

Kodihalli S, K. D., Webster RG (2000). "Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines." Vaccine **18**(23): 2592-9.

La Montagne JR, N. G., Quinnan GV, Curlin GT, Blackwelder WC, Smith JI, Ennis FA, Bozeman FM (1983). "Summary of clinical trials of inactivated influenza vaccine - 1978." Rev Infect Dis **5**(4): 723-36.

Leneva IA, R. N., Govorkova EA, Goloubeva OG, Webster RG (2000). "The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses." Antiviral Res **48**(2): 101-115.

Lin YP, S. M., Gregory V, Cameron K, Lim W, Klimov A, Subbarao K, Guan Y, Krauss S, Shortridge K, Webster R, Cox N, Hay A (2000). "Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates." Proc Natl Acad Sci USA **97**(17): 9654-8.

Maassab HF, F. T. J., Davenport FM, et al. (1969). "Laboratory and clinical characteristics of attenuated strains of influenza virus." Bull World Health Organ **41**: 589-94.

Masurel N, M. W. (1973). "Recycling of Asian and Hong Kong influenza A virus hemagglutinins in man." Am J Epidemiol **97**: 44-9.

Matrosovich M, T. A., Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I,

(H3N2) epidemic." J Med Microbiol **49**(6): 553-556.

Deguchi Y, T. Y. (2000). "Efficacy of influenza vaccine in the elderly: reduction in risks of mortality and morbidity during an influenza A (H3N2) epidemic for the elderly in nursing homes." Int J Clin Lab Res **30**(1): 1-4.

Dolin R, R. R., Madore HP, Maynard R, Linton PN, Webber-Jones J (1982). "A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection." N Engl J Med **307**: 580-584.

Donnelly JJ, F. A., Martinez D, Montgomery DL, Shiver JW, Motzel SL, Ulmer JB, Liu MA (1995). "Preclinical efficacy of a prototype DNA vaccine: Enhanced protection against antigenic drift in influenza virus." Nat Med **1**(6): 583-587.

F, S. K. (1995). "The next pandemic influenza virus?" Lancet **346**: 1210-1212.

Fanning TG, R. A., Taubenberger JK (2000). "Influenza A virus neuraminidase: regions of the protein potentially involved in virus-host interactions." Virology **276**(2): 417-423.

Fanning TG, T. J. (1999). "Phylogenetically important regions of the Influenza A H1 hemagglutinin protein." Science **65**(1): 33-42.

FG, H. (2000). "Influenza virus and rhinovirus-related otitis media: potential for antiviral intervention." Vaccine **19**(Suppl 1): S66-S70.

Foster DA, T. A., Furumoto-Dawson A, et al. (1992). "Influenza vaccine effectiveness in preventing hospitalization for pneumonia in the elderly." Am J Epidemiol **136**: 296-307.

Glezen, W. P. (1996). "Emerging Infections: Pandemic Influenza." Epidemiologic Reviews **18**(1): 64-74.

Gorman OT, B. W., Kawaoka Y, Webster RG. (1990). "Evolution of the nucleoprotein gene of influenza A virus." J Virol **64**: 1487-1497.

Gorse GJ, B. R., Munn NJ. (1991). "Superiority of live attenuated compared with inactivated influenza A virus vaccines in older, chronically ill adults." Chest **100**(977-984).

Gross PA, H. A., Sachs H, Levandowski RA (1995). "The efficacy of influenza vaccine in the elderly: a meta-analysis and review of the literature." Ann Intern Med **123**: 518-527.

Group, T. M. S. (1998). "Randomized trial of efficacy and safety of inhaled zanamivir in treatment of influenza A and B virus infections." Lancet **352**: 1877-1881.

Guan Y, S. K., Krauss S, Li PH, Kawaoka Y, Webster RG (1996). "Emergence of avian H1N1 influenza viruses in pigs in China." J Virol **70**: 8041-8046.

Gubareva LV, K. L., Hayden FG (2000). "Influenza virus neuraminidase inhibitors." Lancet **355**(9206): 827-35.

Hayden FG (1994). "Amantidine and rimantadine resistance in influenza A viruses." Curr Opin Infect Dis **7**: 674-77.

Hayden FG, A. R., Schiling M, et al. (1999). "Use of the selective oral neuraminidase in

Kawaoka Y (2000). "Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals." J Virol **74**(18): 8502-12.

McCullers Jonathan A, F. S., Chesney P. Joan, Webster Robert G (1998). "Influenza B Virus Encephalitis." CID: 898-900.

Monto AS, A. N. (1992). "Implications of viral resistance to amantadine in control of influenza A." Clin Infect Dis **15**: 362-367.

Monto AS, R. D., Herlocher ML, Hinson JM, Elliott MJ, Crisp A (1999). "Zanamivir in the prevention of influenza among healthy adults: a randomized controlled trial." JAMA **282**: 31-35.

Mori SI, N. M., Sasaki Y, Mori K, Tabei Y, Yoshida Y, Yamazaki K, Hirata I, Sekine H, Ito T Suzuki S (1999). "A novel amino acid substitution at the receptor-binding site on the hemagglutinin of H3N2 influenza A viruses isolated from 6 cases with acute encephalopathy during the 1997-1998 season in Tokyo." Arch Virol **144**: 147-155.

NcNicholl IR, M. J. (2001). "Neuraminidase inhibitors: zanamivir and oseltamivir." Ann Pharmacother **35**(1): 57-70.

Nichol KL, M., PM, Mallon, KP, Jackson, LA, Gorse, GJ, Belshe, RB, Glezen, WP, Wittes, J (1999). "Effectiveness of Live, Attenuated Intranasal Influenza Virus Vaccine in Healthy, Working Adults." JAMA **282**(2): 137-144.

Nichol KL, M. K., Wuorenma J, et al. (1994). "The efficacy and cost effectiveness of vaccination against influenza among elderly persons living in the community." N Engl J Med **331**: 778-84.

Nichol KL, M. K., Wuorenma J, et al. (1995). "The effectiveness of vaccination against influenza in healthy, working adults." N Engl J Med **333**: 889-893.

Nicholson KG, A. F., Osterhaus AD, Trottier S. Carewicz O, Mercier CH, Rode A, Kinnersley N, Ward P (2000). "Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group." Lancet **355**(9218): 1845-50.

Oates, J. A., Wood, Alastair, J.J. (1990). "Drug Therapy." The New England Journal of Medicine **322**(7): 443-450.

O'Brien KL, W. M., Sellman J, Quinlisk P, Regnery H, Schwartz B, Dowell SF (2000). "Severe pneumococcal pneumonia in previously healthy children: The role of preceding influenza infection." Clinical Infect Dis **30**: 784-789.

Offringa DP, T.-M. V., Ye Z, Levandowski RA (2000). "A comprehensive systematic approach to identification of influenza A virus genotype using RT-PCR and RFLP." J Virol Methods **88**(1): 15-24.

Ohmit SE, M. A. (1995). "Influenza vaccine effectiveness in preventing hospitalization among the elderly during influenza type A and type B seasons." Int J Epidemiol **24** (1240-1248).

Oshitani H, S. R., Seki N, Tanabe N, Yamazaki O, Hayashi S, Suzuki H (2000).

"Influenza vaccination levels and influenza-like illness in long-term-care facilities for elderly people in Niigata, Japan, during an influenza A (H3N2) epidemic." Infect Control Hosp Epidemiol **21**(11): 728-730.

Osterhaus, A., Rimmelzwaan, GF, Martina, BEE, Bestebroer, TM, Fouchier, RAM (2000). "Influenza B Virus in Seals." Science **288**: 1051-1053.

Parkman PD, H. H., Rastogi SC, et al. (1977). "Summary of clinical trials of influenza virus vaccines in adults." J Infect Dis **136 (suppl)**: S722-30.

Poland, G., Couch, R (1999). "Intranasal Influenza Vaccine Adding to the Armamentarium for Influenza Control." JAMA **282**(2): 182-184.

Powers DC, F. L., Murphy BR, Thumar B, Clements ML (1991). "In elderly persons live attenuated influenza A virus vaccines do not offer an advantage over inactivated virus vaccine in inducing serum or secretory antibodies or local immunologic memory." J Clin Microbiol **29**(3): 498-505.

RB., C. (1993). "Advances in influenza virus vaccine research." Ann N Y Acad Sci **685**: 803-812.

Reichert, T. A., Sugaya, Norio, Fedson, David, Glezen W. Paul, Simonsen, Lone, Tashiro, Masato (2001). "The Japanese Experience with Vaccinating Schoolchildren Against Influenza." The New England Journal of Medicine **344**(12): 889-895.

Reid AH, F. T., Hultin JV, Taubenberger JK (1999). "Origin and evolution of the 1918 'Spanish' influenza virus hemagglutinin gene." Proc Natl Acad Sci USA **96**: 1651-1656.

Reid AH, F. T., Janczewski TA, Taubenberger JK (2000). "Characterization of the 1918 'Spanish' influenza virus neuraminidase gene." Proc Natl Acad Sci USA **97**: 6785-6790.

Reid AH, T. J. (1999). "The 1918 flu and other influenza pandemics: 'Over there' and back again." Lab Invest **79**: 95-101.

RG., D. (1990). "Drug therapy: prophylaxis and treatment of influenza." N Engl J Med **322**: 443-450.

Rodriguez WJ, H. C., Welliver R, et al. (1994). "Efficacy and safety of aerosolized ribavirin in young children hospitalized with influenza: a double-blind, multicenter, placebo-controlled trial." J Pediatr **125**: 129-135.

Roos-van Eijndhoven, D. G., Cools, H.J.M., Westendorp, R.G.J., Cate-Hoek, A.J. Ten, Knook, D.L., Remarque, E.J. (2001). "Randomized Controlled Trial of Seroresponses to Double Dose and Booster Influenza Vaccination in Frail Elderly Subjects." Medical Virology **63**(4): 293-298.

Schafer JR, K. Y., Bean WJ, Suss J, Senne D, Webster RG (1993). "Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir." Virology **194**(2): 781-788.

Schoenbaum SC, C. M., Dowdle WR, Mostow SR (1976). "Epidemiology of influenza in the elderly: evidence of virus recycling." Am J Epidemiol **103**: 166-73.

Scholtissek C, L. S., Fitch WM (1993). "Analysis of influenza A virus nucleoproteins for

the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages." Arch Virol **131**: 237-250.

Schonberger LB, B. D., Sullivan-Bolyai JZ, et al. (1979). "Guillain-Barre syndrome following vaccination in the National Influenza Immunization Program, United States, 1976-1977." Am J Epidemiol **110**: 105-123.

Shortridge KF, S.-H. C. (1982). "An influenza epicentre?" Lancet **2**: 812-813.

Simonsen L, C. M., Schonberger LB, Arden NH, Cox NJ, Fukuda K (1998). "Pandemic versus epidemic influenza mortality: A pattern of changing age distribution." J Infect Dis **178**: 53-60.

Skoner DP, G. D., Patel A, Doyle WJ (1999). "Evidence for cytokine mediation of disease expression in adults experimentally infected with influenza A virus." J of Infect Dis **180**: 10-14.

Steinhauer, D. A. (1999). "Role of Hemagglutinin Cleavage for the Pathogenicity of Influenza Virus." Virology **258**: 1-20.

Taubenberger, J., Reid, AH, Krafft, AE, Bijwaard, KE, Fanning, TG (1997). "Initial Genetic Characterization of the1918 "Spanish" Influenza Virus." Science **275**: 1793-1796.

Taubenberger, J. (1999). "Seeking the 1918 Spanish influenza virus." ASM News **65**(7): 473-478.

Taubenberger, J. (2000). "The 1918 Influenza Virus: A Killer Comes into View." Virology **274**: 241-245.

Togashi T, M. Y., Narita M (2000). "Epidemiology of influenza-associated encephalitis-encephalopathy in Hokkaido, the northernmost island of Japan." Pediatrics International **42**: 192-196.

Treanor JJ, B. R. (1998). "Evaluation of live, cold-adapted influenza A and B virus vaccines in elderly and high-risk subjects." Vaccine **16**(18): 1756-60.

Treanor JJ, H. F., Vrooman PS, et al. (2000). "Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial." JAMA **283**(8): 1016-24.

Voeten JT, B. R., Palache AM, van Scharrenburg GJ, Rimmelzwaan GF, Osterhaus AD, Claas EC. (1999). "Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium." Vaccine **17**(15-16): 1942-50.

Voeten JT, C. E., Brands R, Palache AM, et al (1999). "Generation and characterization of reassortant influenza A viruses propagated in serum-free cultured MDCK-SF1 cells." Dev Biol Stand **98**: 77-90.

Webster RG, B. W., Gorman OT, Chambers TM, Kawaoka Y (1992). "Evolution and ecology of influenza A viruses." Microbiol Rev **56**: 152-179.

Webster RG, L. W., Tumova, B (1975). "Studies on the Origin of Pandemic Influenza Viruses." Virology **67**: 534-543.

Whitley RJ, H. F., Reisinger KS, Young N, Dutkowski R, Ipe D, Mills RG, Ward P (2001). "Oral oseltamivir treatment of influenza in children." Pediatr Infect Dis J **20**(2): 127-133.

WP, G. (2000). "Prevention of acute otitis media by prophylaxis and treatment of influenza virus infections." Vaccine **19**(Suppl 1): S56-S58.

WR, D. (1999). "Influenza A virus recycling revisited." Bull WHO **77**(10): 820-8.

Zakstelskaja LJ, Y. M., Isacenko VA, et al. (1978). "Influenza in the USSR in 1977: recurrence of influenza virus A subtype H1N1." Bull WHO **56**: 919-922.