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Review article

Current Research on Influenza and other Respiratory Viruses: II International Symposium

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1. Introduction

The Second International Symposium on Influenza and Other Respiratory Viruses was convened by The Macrae Group (New York City, NY) in Grand Cayman, Cayman Islands on 10–12 December 1999. A summary of the First International Symposium was published in the Journal of Antiviral Research (Kaiser et al., 1999). The purpose of the Second Symposium was to reunite leading experts from around the world to review the most recent developments in research on influenza, respiratory syncytial, and other respiratory viruses, and to provide new insights into their epidemiology, clinical impact, detection, pathogenesis, and management. Novel approaches to immunization and antiviral treatment were emphasized. The meeting was intended to benefit all those working in the field by providing a multidisciplinary forum for open discussion among representatives from academia, government and industry.

The meeting was chaired by Frederick G. Hayden, University of Virginia, USA and Robert B. Couch, Baylor College of Medicine, USA. It was attended by participants from 18 countries. Invited speakers included: Goran Wadell, The University Hospital, Umea, Sweden; Per Ljungman, Huddinge Hospital, Sweden; Marie Griffin, Vanderbilt University School of Medicine, Nashville, TN, USA; Robert Webster, St. Jude

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Children's Research Hospital, Memphis, TN, USA; Nancy Cox, Centers for Disease Control and Prevention, Atlanta, GA, USA; Alan Hampson, WHO Collaborating Centre for Influenza Reference and Research, Parkville, Vic., Australia; Dominick Iacuzio, Hoffman La Roche, Nutley, NJ, USA; Daniel Lavanchy, WHO, Geneva, Switzerland; Linda Lambert, NIAID, National Institutes of Health, USA; Jefferey Taubenberger, Armed Forces Institute of Pathology, Washington, DC, USA; Annika Linde, Swedish Institute for Infections and Disease Control, Stockholm, Sweden; Jack Gwaltney Jr., University of Virginia, Charlottesville, VA, USA; Tasnee Chonmaitree, University of Texas Medical Branch, Galveston, TX, USA; Peter Palese, Mount Sinai School of Medicine, New York, NY, USA; Albert Osterhaus, Erasmus Universiteit Rotterdam, The Netherlands; Robert Belshe, Saint Louis University, Saint Louis, MO, USA; Paul Glezen, Baylor College of Medicine, Houston, TX, USA; Pedro A. Piedra, Baylor College of Medicine, USA; Brian Murphy, NIAID, NIH, USA; Peter Wright, Vanderbilt University School of Medicine, Nashville, TN, USA; Edwin Kilbourne, New York Medical College, Valhalla, USA; Peter Colman, Biomolecular Research Institute, Parkville, Vic., Australia; Janet McElhaney, Eastern Virginia Medical School, Norfolk, USA; Diane Young, Pharmaceutical Research Institute, NJ, USA; Noel Roberts, Roche Products, Welwyn Gadek City, UK; Frederick Hayden, University of Virginia, Charlottesville, USA; and Olli Ruuskanen, University of Turku, Finland. Submitted abstracts provided the basis for 12 late breaker oral presentations and a poster session.

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2. Epidemiology and impact of respiratory viruses

2.1. Influenza surveillance in animals: implications for humans

Type A influenza virus is an RNA virus with a negative sense segmented genome. It exists as quasi species with no proof reading mechanisms so it is prone to mutate during replication. These properties make it a highly variable virus that undergoes continuous antigenic drift (minor antigenic variation) and infrequent antigenic shift (major antigenic variation). Fifteen hemagglutinin (H1–H15) and nine neuraminidase (N1–N9) subtype antigens of influenza A virus have been recognized in nature, with all subtype antigens occurring in wild bird reservoirs, particularly ducks and shorebirds. There are two clades of animal influenza viruses, the Eurasian and the American.

In 1998, highly transmissible influenza A (H3N2) strains that resulted in significant morbidity (high fever, fetal abortion) and variable mortality appeared in swine in the US. Two genetically distinct H3N2 swine virus variants have been identified (Zhou et al., 1999), one is a double reassortant originating from a human H3N2 and a pig H1N1 strain. The second is a triple reassortant with proteins derived from human H3N2 (PB1, HA, NA), swine H1N1 (NP, M, NS), and American avian (PB2, PA) strains. This latter virus is currently widespread in pig raising regions of the US and could potentially reinfect humans.

Viruses in chicken reservoirs are also undergoing important changes. In Italy, in 1995, a prevalent and previously innocuous H3N2 variant began to cause disease in chickens. In 1997–1998, another subtype, H5N1 virus, appeared in Hong Kong poultry markets and in humans, causing respiratory disease in humans severe enough to result in the death of six of 18 persons infected. Since the implementation of major measures of control that included the slaughter of poultry and extensive cleaning of the markets, the H5N1 virus has not been detected again in Hong Kong.

In 1999, a H9N2 virus emerged in China causing increased morbidity and mortality of chickens and decreased egg production. Another lineage of H9N2 detected in two children with respiratory disease was indistinguishable from A/Quail/Hong Kong/G1/97 (H9N2) viruses that comprise one-third of influenza viruses being isolated from poultry markets in Hong Kong. This virus contains 6 genetic segments that are similar to those of H5N1 virus, providing the potential for this virus to transmit to humans (Guan et al., 1999).

Until now, influenza B virus has not been known to have an animal reservoir. However, a variant of influenza B virus (B/Seal/Netherlands/1/99) was recently isolated from a throat swab from a harbor seal (*Phoca vitulina*) with respiratory symptoms admitted to the Rehabilitation and Research Center in Pieterburen, Netherlands. Serologic evidence of infection (IgM and IgG hemagglutinin-inhibition antibody) was documented in this and another contact animal. The genetic sequence of the virus was homologous with the human epidemic influenza B strains circulating in 1995–1998. Additionally, serologic evidence of influenza B infection was documented in eight of 600 seals sampled after 1995. Although the mechanism of transmission remains unknown, this is the first time a human influenza B virus has been found to have caused natural infection in an animal species. The capacity of seals as reservoirs of influenza viruses remains to be determined.

Interspecies transmission of influenza A viruses has been occurring for years, but chimeric viruses appear to be occurring more frequently. Whether this represents increased occurrence or increased recognition is not certain. Contributing factors for an increased occurrence include the increase in animal and human populations and in opportunities for interaction. The concern is the potential for increased interspecies transmission that could raise the possibility of emergence of new pandemic strains for humans.

2.2. Influenza in children and the elderly

During the 1998–1999 influenza epidemic in Houston, TX, influenza was diagnosed by culture or enzyme immunoassay (EIA) in 584 children with upper or lower respiratory tract symptoms (influenza A in 83%, Influenza B in 17%). Children under 5 years of age (particularly if under 1 year of age) had the highest rates of hospitalization for acute respiratory disease and laboratory confirmed influenza. Children with underlying conditions that placed them at high risk for complications of influenza experienced greater morbidity and more frequent hospitalization; however, 22% of hospitalized children had no risk factors. If under 5 years of age, these children were more likely to develop lower respiratory tract disease, otitis media, febrile illnesses and other complications such as febrile seizures, gastroenteritis and dehydration. Vaccination rates were low ($<5\%$) in high risk children, and nil in previously healthy children.

During influenza epidemics there is an increased death rate among the elderly. In a study of respiratory infections in the elderly in Stockholm, Sweden, influenza was found to be the predominant pathogen. Between January 1997 and June 1999, 539 respiratory samples were obtained from ill patients 65 years of age or older. Viral pathogens included: influenza A (52%); influenza B (4%); respiratory syncytial virus (RSV) (1.3%); and parainfluenza virus (PIV) (0.7%). The impact of influenza was also shown to be important in long term care facilities in Ontario, Canada, where 416/844 (49%) long term care facilities experienced an outbreak of respiratory disease between July 1998 and June 1999. Of these, 51% were due to influenza alone, 7% to RSV alone, 5% to influenza and RSV, 3% PIV, 3% rhinovirus (RV), and 1% to other agents. Facilities with higher influenza vaccination rates of staff reported fewer outbreaks.

2.3. Pandemic influenza, are we prepared?

Currently, it is not possible to accurately predict the time of appearance of the next influenza pandemic. Thus, planning for a pandemic is a process that involves developing strategies and building expertise and infrastructures to deal with an unpredictable and perhaps catastrophic event, and anticipating from past experiences the possible unexpected consequences of certain strategies and interventions. Planning should accomplish two objectives: (1) effective assessing the pandemic risk;

and (2) define the role and possible actions of the various medical and public health institutions. Pandemic preparedness was addressed by a panel of experts.

2.3.1. Role of the World Health Organization (WHO)

The roles of the WHO are to maintain a global influenza surveillance network that will facilitate the detection of novel influenza viruses during interpandemic periods, and to declare the onset and end of a pandemic. Significant collaboration worldwide is necessary to accomplish this. National health authorities in all countries should establish a pandemic planning committee for decision making regarding surveillance, scientific and medical issues, supply and distribution of antiviral drugs, and management of the pandemic, taking into consideration communication, legal, political and economical issues (JID, 1997). Countries should make the best use of available resources to reduce the impact of disease. Management of exposure as an approach to prevention of a pandemic is not possible because of the current high levels of international travel and the expansion of populations in many regions of the world.

2.3.2. Influenza surveillance

The purposes of influenza surveillance include: to know which influenza viruses are circulating; to determine the impact of influenza activity; to detect unusual events such as emergence of a pandemic strain; to assist in disease control with surveillance-based recommendations; and to determine the effectiveness of control measures. Early detection of new influenza strains is crucial for a rapid response to a pandemic threat. For recent influenza pandemics, a gap of several months occurred between the time that widespread outbreaks of influenza were occurring and the time the virus was first isolated. This was true for the 1957 pandemic, the 1968 pandemic and for the H1N1 virus in 1977. Many surveillance lessons were learned from the experience with the H5N1 outbreak in 1997 (Snacken et al., 1999). In this case, the WHO network identified a single isolate of H5N1 early in the process, allowing for local preparations, but the need for better surveillance in Asia, particularly in China, was apparent. We learned how difficult it is to enhance global and even national surveillance and that there is a great need for expanded influenza surveillance in animals. We learned that influenza viruses can jump the host species barrier without reassortment and that there is a need to have an increased availability of biosafety level 3 containment facilities worldwide to study highly virulent viruses. The need for training for surveillance became obvious as well.

In many countries, the funding for influenza surveillance is very limited. New and innovative mechanisms need to be in place to provide funding for surveillance. A contingency plan for enhancing global surveillance during a pandemic alert needs to be developed and expansion of influenza surveillance in China and other pacific basin countries is necessary.

2.3.3. Vaccination

Interventions for control of pandemic influenza can be considered at three levels: (1) to prevent all or some of the population from becoming infected; (2) to limit or at least slow the progress of the epidemic; and (3) to deal with the consequences of infection. The elimination of the source, as occurred with the slaughtering of poultry during the H5N1 outbreak in Hong Kong, is unlikely to be achieved if the virus transmits efficiently from person to person. The options for slowing spread include the use of antiviral prophylaxis, limiting congregations of people, and possibly quarantine. Antiviral prophylaxis is not the best approach at the present time because of the need to have an adequate supply for a very large population for many weeks.

Providing effective vaccination should be the principal focus of a pandemic plan (Cox and Fukuda, 1998). Practical issues to consider include how to provide a vaccine supply quickly and to optimally use available doses. There are often delays in the preparation of a vaccine from the time the virus is first identified to the time the vaccine is ready for distribution. The WHO plan considers distribution of candidate vaccines and many national plans list vaccine as an intervention, but do not have a plan for obtaining it. If all candidate virus subtypes were prepared in advance, vaccine

production could be shortened to 11 or 12 weeks, compared with the normal 22–23 week process. Nevertheless, there are major questions to be answered: how much antigenic variation is there within each subtype? Are the current virus subtype strains representative of all the candidates? How much and who will cover the costs of preparing candidate vaccines and how would we select the variants for stock piling and vaccine preparation? Since the world population would largely be susceptible to a new subtype, one possibility would be to use the most antigenically similar available strain to obtain a degree of immunologic priming within that subtype and a decrease in the severity of disease in these immunized individuals. An alternate mechanism would be to use recombinant live attenuated viruses that may induce a broader spectrum of immune responses than inactivated vaccine. At this time, it is possible to prepare these various subtype reagents knowing that some might not be used.

Since the last pandemic of 1969, the method of preparing vaccines has changed, limiting comparisons with responses observed in previous studies. Split virus vaccines are now made instead of whole virus vaccines, and the responses to these may be different. However, there is an increased production capacity. The amount of protection conferred by different vaccines and the minimum effective dose for protection need to be considered. Options include giving one dose to the entire population or two doses to half the population, assuming there are enough doses to achieve this. During the 1976–1977 New Jersey outbreak, children that were naïve to the virus had a better response to whole virus than to split virus vaccines, but two doses of whole virus vaccine were needed to achieve protective levels of antibody.

Cell culture based vaccines have been considered and investigated for a number of years but have not yet entered general use. The advantage of MDCK cells is that they can grow in suspension, but the disadvantage of these and other cells such as Vero is that vaccine production requires that their growth be adapted to serum free conditions and addition of trypsin for efficient replication. Although there are approved vaccines grown in Vero cells (polio), the preferred cells for influenza would probably be MDCK cells, and these would have to undergo further regulatory considerations such as determining the presence of adventitious viruses, prions, and nucleic acids. Other issues to consider are the production capacity of these cell lines and economic issues of using this source. They have about the same development time as egg grown vaccines but they are potentially more scalable.

Alternative vaccines can be considered. Live attenuated vaccines will require approximately the same production time as inactivated vaccines or longer because of a need for establishing safety and possibly doing dose ranging studies; moreover, they require special pathogen free eggs which are in limited supply. Genetically engineered hemagglutinin gave poor results with the H5N1 subtype in humans at doses of 10–20 μg. DNA vaccines and addition of an adjuvant to vaccines are other options for the future.

2.3.4. Antivirals

While the first line of defense against a pandemic is vaccination, antiviral drugs are an alternative. Amantadine and rimantadine are approved for the treatment and prophylaxis of influenza A. Their effectiveness against pandemic influenza has been proven in earlier trials. In 1999, a new class of antiviral agents with activity against both influenza A and B became available; the neuraminidase inhibitors (NI) zanamavir (Relenza®–Glaxo) and oseltamivir (Tamiflu®–Roche) are now approved for use in various countries in Europe and North and South America. The utility of these drugs during a pandemic needs to be considered.

The current antiviral drugs in the US are available in a limited number of doses (less than 1.5 million doses) that would be rapidly depleted in the case of a pandemic. As indicated earlier, prophylaxis of a large number of people is not an option. Only one in 35 patients currently receive an antiviral drug for treatment of influenza. Less than 1.1 million prescriptions were written during the 1998–1999 influenza season. To assess the potential contribution of antivirals, the current and future production capacity, purchasing and distribution logistics, and funding need to be determined. Each manufacturer has its own process, timelines, steps,

and rate limiting stages for production. However, the potential to stock-pile drugs exists.

It is currently recommended that antivirals not be used for animal influenza, including pigs, horses and chickens, because of the potential induction of a resistant pandemic strain. Options need to be discussed with those involved in swine and poultry production to coordinate longitudinal animal and human studies needed to learn which viruses are circulating in animal populations, the frequency at which they are transmitted to humans, and the properties of those transmitted viruses. Antiviral intervention in animals might reduce the risk of generation of a new pandemic virus.

In the US, current options for the optimal use of existing antivirals during a pandemic include: government purchase of drug for high risk groups; use of existing supplies for prophylaxis; and treatment of high risk groups and severely ill patients. Early treatment offers the benefit of increased chance of therapeutic success, reduction of complications, no interference with immunity, and a potential for decrease of viral transmission. It is therefore important to consider and develop strategies for production and priorities for distribution of each class of antiviral drugs.

2.3.5. Role of NIH in support of research

In 1995, the NIAID convened an international workshop to consider influenza pandemic preparedness and to identify critical gaps or issues (JID, 1997). Recommendations developed by the pandemic planning committees included: (1) expanding surveillance; (2) developing a library of high-growth reassortants of different influenza subtypes; (3) producing and clinically testing pilot lot vaccines from avian strains with pandemic potential; (4) establishing a cell culture system for production of influenza vaccines; (5) increasing collaboration among international laboratories; (6) sharing of information, reagent strains, and new technological advances; and (7) training for laboratory and clinical research.

One of the main goals set during the workshop was to improve international surveillance efforts with an emphasis on animal surveillance. A multi-year contract was awarded to St Jude's Research Hospital for work on this issue. In addition to surveillance and identification of isolates, viruses could be made available to the community should they become important in vaccine development. Another goal was to prepare a complement of reference reagents for identifying and working with new hemagglutinin (HA) and neuraminidase (NA) subtypes. Purified proteins, monoclonal antibodies and sheep antisera are being prepared; a new H9 protein sheep antiserum will soon be available.

The use of cold adapted trivalent influenza vaccine in children to alter the course of an outbreak is currently being evaluated in Texas by investigators at Baylor College of Medicine. Support is being provided for other clinical trials, including a purified NA supplemented inactivated vaccine. Improvement in the understanding of the role of humoral, cellular and mucosal immunity in protection against influenza has been stimulated through the development of a series of RO1 investigator initiated research grants. NIAID is also supporting basic biology, molecular epidemiology, and pathogenesis of influenza as well as research on adjuvants and novel delivery systems.

2.4. Respiratory viruses in selected patient populations

2.4.1. Respiratory viruses in transplant patients

Assessment of the true impact of respiratory viruses in transplant patients has been difficult due to the lack of controlled studies. Current reports represent data on selected populations, particularly those who suffer the most severe infections. Therefore, a causal relationship between viral infection and outcome can not be clearly determined.

In a prospective study of 2000 bone marrow transplant (BMT) recipients in 36 European centers, the incidence of respiratory viral infections was 2.7%. Allogeneic stem cell transplant (STC) recipients had a higher frequency (4.7%, range 0–18.4%), than autologous STC recipients (0.4%, range 0–17.4%); the outcome of the respiratory virus infection was, however, similar in both groups (76 and 74% survival, respectively).

RSV is a cause of serious disease in BMT recipients. Upper respiratory tract infection (URTI) progresses to pneumonia in 20% or more patients. Survival after RSV URTI is approximately 92%, but decreases to only 49% if pneumonia develops. Infection can occur early (within 2 months) or late after transplantation. Late infection begins as a mild URTI, which commonly progresses to obstructive bronchiolitis and chronic oxygen dependency. Worsening respiratory symptoms and respiratory insufficiency can continue even after RSV is cleared from the respiratory tract.

The time of highest risk of infection with RSV in BMT patients is variable. According to studies at MD Anderson Hospital in Texas, during the period of neutropenia (early phase) up to 80% of RSV URTI progress to pneumonia, but this occurs rarely after engraftment (Whimbey et al., 1996). In recent European studies, neutropenia was not a risk factor for progression to lower respiratory tract disease. Older age, lymphopenia and unrelated or HLA mismatched BMT recipients were at higher risk for the development of RSV pneumonia.

Anecdotal treatment regimens for RSV disease in the BMT patient population have included the administration of ribavirin alone or in combination with immunoglobulin G (IgG). Although treatment with this combination remains under investigation, it appears to provide some benefit. The route of administration of ribavirin has also been studied. In Europe, the treatment combination of intravenous (IV) and inhaled (IH) ribavirin with or without IgG has resulted in improved survival of affected patients compared with those treated with IV or IH ribavirin alone or in combination with IgG. The mechanism of this potential effect and the optimal routes of administration still need to be defined. Monoclonal antibody against RSV for the treatment of patients with pneumonia has been studied in a small group of patients at high risk; 12 of 15 (80%) patients survived.

Parainfluenza virus infection has been detected in up to 5% of BMT recipients in Texas (Lewis et al., 1996). In a group of 61 patients with PIV, 44% developed pneumonia and 37% died (overall mortality of 16%). PIV also causes more severe disease in patients receiving transplants from unrelated mismatched donors and is an independent cause of death in allogeneic stem cell transplant recipients. In European studies, PIV respiratory tract infection in 21 BMT patients resulted in mortality as high as 40%. Treatment regimens have included IV or IH ribavirin; however, the small numbers of subjects in these reports and the number of PIV serotypes limit interpretation of these data.

Influenza virus infection in BMT recipients frequently causes interstitial pneumonia and is associated with a rate of mortality of approximately 20% overall (Whimbey et al., 1994). In European studies, an overall survival of 90% has been documented. The period of greatest risk for the development of pneumonia is more than a year after transplantation. Treatment with amantadine or rimantadine has been used but there are no data on their efficacy in this patient population.

BMT recipients have a poor response to influenza vaccine. Attempts to improve this response by the administration of granulocyte and monocyte colony stimulating factors have resulted in no additional benefit in allogeneic BMT recipients and in autologous BMT recipients with hematologic malignancies, but improved responses in breast cancer patients occurred when given less than 12 months after the transplant.

Rhinovirus has been associated with fatal pneumonia and ARDS in transplant patients (Ghosh et al., 1999). In European studies, five deaths occurred in 11 patients diagnosed with RV infection, but a cause-effect relationship was not found. There are no other data that would support the association of RV infection with severe outcome in BMT recipients at this time.

2.4.2. Respiratory viruses in HIV

Three studies were summarized that support the hypothesis that human immunodeficiency virus (HIV) infected patients have more frequent and/or more severe infections due to respiratory viruses. One small trial with inactivated influenza virus vaccine demonstrated good efficacy, but data is lacking on both burden of disease and efficacy of immunization by level of immunodeficiency.

Infants ≤4 months of age were enrolled in a prospective cohort study and followed for up to 2.5 years for the development of febrile upper and lower respiratory tract infections (King et al., 1993). HIV-infected children had higher rates of both endpoints (335 and 49 per 100 person-years, respectively) than matched children without HIV infection (132 and 19 per 100 person-years). Documented viral infections accounted for 37% of all illnesses with RSV isolated in 35% of these. RSV-infected children with HIV had prolonged RSV viral shedding, more frequent pneumonia, and less frequent bronchiolitis than did children without HIV infection.

A significant increase of deaths from pneumonia and influenza (P&I) among persons aged 25–44 was noted in the US beginning in 1981. Investigation of this phenomenon revealed that the increase was greatest in cities with a higher incidence of AIDS. In addition, the P&I mortality rates peaked in winter months suggesting a role for influenza and/or other respiratory viruses.

A retrospective cohort study of women aged 15–64 (1.7 million person-years) enrolled in Tennessee's Medicaid program reported that HIV-infected women had a risk of influenza-attributable hospitalizations at least as high as women with other high risk medical conditions (Neuzil et al., 1999). However, the estimate of 33 (95% CI 7–59) acute cardiopulmonary hospitalizations per 1000 HIV-infected women was based on relatively small numbers (400 person years).

The response of HIV infected patients to influenza vaccine was studied in a group of HIV infected military recruits during the 1995–1996 influenza season (Tasker et al., 1999). A poor response to vaccine was observed despite the lack of advanced HIV disease. Nevertheless, the vaccine efficacy against influenza A URTI was very good; 16/55 (29%) of vaccinated recruits reported a URTI versus 23/47 (49%) of placebo recipients, suggesting that illness was prevented in 20% of vaccine recipients. Influenza virus was isolated in five of 12 placebo recipients and zero of eight symptomatic immunized individuals providing a vaccine efficacy against influenza A infection of 93% (range 69–100%).

3. Virology and detection

3.1. Characterization of the 1918 influenza virus: application of genomics

The 1918 influenza virus pandemic killed millions of people in a period of 1 year. The first wave of the pandemic, reported as early as March 1918, was present simultaneously in the Far East, Europe and the US. Over the summer months of 1918, the virus ravaged troops in Europe on both sides of the front; in the fall of 1918 and early part of 1919 it exploded into a virulent second wave on all continents. Conservative estimates have attributed 20 million deaths to influenza, but more recent estimates are closer to 40 million. In the US there were about 550 000 excess deaths and 675 000 total deaths. In Philadelphia there were 16 000 deaths of which 11 000 occurred in October, and in the US military, 43 000 of 100 000 total troop casualties during World War I were attributable to influenza. The life expectancy in the US in the 20th century had a precipitous drop of 13 years in 1918. That drop occurred because of the unusual age adjusted mortality with a unique peak in the 15–45 year age group.

Importantly, about 97% of the people who had influenza in 1918 had a 3-day course of fever and improved. Of the 2–3% who died, the main cause was a secondary bacterial pneumonia. A subset of victims, usually young adults, died very rapidly, sometimes within 24–48 h of onset, and exhibited a distinct pathology characterized by massive pulmonary edema and hemorrhage. This was also seen in a small percentage of patients in the 1957 pandemic but it was more frequent in 1918.

The goal of the project that seeks to sequence the 1918 virus is to determine where the virus came from, where it went, and why it was so virulent. RNA has been recovered from lung tissue of victims of the 1918 pandemic. The first case was a healthy 21-year-old soldier from Fort Jackson, South Carolina, who had a 5 day course of influenza at the beginning of the peak of the second wave and died on the 26th of September of a massive bacterial pneumonia of the left lung. The second case was that of a 30-year-old healthy male who had influenza in September in an army

camp in New York, and died of massive pulmonary edema after a 3-day illness. The third case represented lung tissue exhumed from the frozen body of an Inuit female (age unknown) who died of influenza in November of 1918 in Northern Alaska. The histology showed massive acute pulmonary hemorrhage.

The fragments of RNA found in these specimens were small pieces of 100–130 bases in length that were representative of the entire genome. The hemagglutinin (HA) gene of the H1 sequence did not have the sequence of basic amino acids at the cleavage site that characterizes virulent avian viruses. Structurally, four potential glycosylation sites were found, as occurs in the consensus avian sequences, but none of the additional glycosylation sites that are seen frequently in human H1 sequences. The antigenic sites were predominantly avian in their consensus sequence and receptor binding site. Of the three cases, there were only two nucleotide differences between the sequences, one of which was a synonymous change and one that was a non-synonymous change at the receptor binding site. The New York case had a glycine at residue 225, which would give it a receptor binding profile similar to swine/Iowa/30 or the European swine H1N1 viruses, with a possible ability for both 2–3 and 2–6 binding. The Alaskan and South Carolina cases had an aspartic acid at that site.

Phylogenetically, the 1918 virus is a mammalian virus that represents an outgroup with avian sequences. This is distinct from the pattern seen in the 1957 and 1968 viruses were the novel HA was clearly avian. Structurally, all the 1918 virus binding sites and glycosylation sites have an avian consensus, suggesting that the HA was an avian H1 that underwent mammalian adaptation. In 1918, the virus was well adapted for humans since it was very infectious, highly transmissible, and replicated at very high copy number in the lungs.

There are two possibilities for the origin of the 1918 virus. One is that the H1 sequence of the 1918 virus was derived from a reassortment event with a bird HA sequence with very few modifications. Avian sequences have drifted more in the last 30 years than previously thought, therefore the 1918 avian sequence would be expected to be very different from modern avian H1 sequences. Within the H1 domain, the nearest avian sequence to the 1918 virus differs by 26 amino acids; in 1957 the nearest avian sequence differs by 15, in 1968 by nine, and in 1987 by one or two amino acids. Drift in avian H1 through the years could have occurred and the 1918 H1 could be from an avian source with very little modification, but represent an avian-like virus that is no longer available. The other possibility is that the avian ancestor of the pandemic virus had been adapting in mammals for a period of time; estimates are for an appearance of the virus around 1910–1915.

The 1918 virus sequence of the neuraminidase (NA) gene is an open reading frame of 1407 bases. It has the seven glycosylation sites characteristic of avian N1 sequences. The N1 antigenic sites have not been identified but 22 antigenic sites have been mapped in N2; seven sites are the same in avian and mammalian sequences, but 15 vary. The 1918 virus has 14 sites that match the avian consensus. The active site of the NA is completely conserved as seen in all other influenza A viruses. Phylogenetic analysis of the NA shows that the N1 sequences are divided into an avian and a mammalian clade. The 1918 sequence is very close to the sequence of the common ancestor of both human and classic swine N1, and originates near the root of the mammalian clade. Like HA, it structurally has many avian features, but phylogenetically is within the mammalian clade. Phylogenetically, NS1 also comes from the root of the human and swine NS sequences.

It has been postulated that the 1918 virus had increased virulence and possibly pantropism or neurotropism. The 1918 virus does not have loss of the glycosylation site at residue 146; it does not have the avian-like cleavage site mutation in the HA, the delta 146 mutation in NA, nor any other structural feature suggesting that it should have behaved in a pantropic manner.

Encephalitis lethargica (of Von Economo) was a neurological complication seen at the time of the 1918 influenza. It was first described by the Austrian neuropathologist Constantin Von Economo in 1917 in patients seen in his clinic in Vienna in 1915–1916. Therefore, the first cases occurred before the outbreak of the Spanish influ-

enza. There were cases that met the description of encephalitis lethargica after 1918, tapering off until 1925 when it disappeared. In that 9-year period there were more than 5 million cases, and it was postulated that this was related to the influenza pandemic and neurotropicity of the virus. The clinical course was varied with a constellation of symptoms that included acute encephalitis with or without death or development of post-encephalitic Parkinson's disease years later.

The archives of the Armed Forces Institute of Pathology were reviewed for evidence of cases of encephalitis lethargica; five cases with an acute death course were identified. There were 70–80 post encephalitic Parkinson's cases, with clinical occurrence decades after the onset of symptoms. Brain blocks were available from which RNA lysates were made. None of the samples were positive for influenza RNA matrix or nucleoprotein. Therefore, in acute encephalitis lethargica deaths with good representation of midbrain and nuclei, influenza RNA was not recovered. However, samples from four coal miners who died in 1918 in Spitsbergen were reported to contain virus RNA in both brain and respiratory tissue. The significance of this finding is not known, but the issue of pantropism was not confirmed after examination of multiple specimens from victims of the 1918 influenza pandemic in the US.

3.2. Progress in practical diagnostics

The possibility for treating influenza has increased the demand for virologic diagnosis. Clinicians vary in their ability to recognize influenza based on clinical manifestations alone. Their ability depends in part on the feedback that they receive from their laboratory when using diagnostic tests. Tests currently available for point of care are rapid kits for antigen detection. These tests have a sensitivity of 45–90% and a specificity of 60–95%, according to the manufacturers. The variation partly depends on the reference method used for evaluation. Other factors include the age of the patient, type of sample, and timing of sample collection relative to the onset of illness. The consequences of poor specificity (false positive) can be worse than that of having a false negative result. Moreover, a spurious interpretation can impair correct labeling of an epidemic or lead to misdiagnosis of another serious infection. Ideally, specificity should be greater than 95%. Other aspects of a rapid diagnostic kit are time for performance and cost.

Antigen detection is currently the method of choice for small to middle sized laboratories. In Sweden, immunofluorescence assays are used. They are feasible as long as the number of samples remains below 20 per day, above that, ELISA assays are more practical. Nasopharyngeal aspirates are used in hospitalized patients but swabs are more commonly used in physician offices. Antigen detection allows for a result in the same day, increasing the interest of the clinician in sending specimens to the laboratory. Local laboratories should be able to store and send samples to central and reference laboratories for epidemiologic purposes. Influenza infectivity is better preserved at 4°C than at freezing temperatures, so specimens should not be frozen unless long-term storage is required.

For central laboratories, antigen detection with ELISA and immunofluorescence are still good methods. Genome amplification is, however, the method of choice in this setting. Virus isolation should also be performed for epidemiological purposes. Reference laboratories should culture, subtype, and sequence viruses, as well as determine antiviral sensitivity. It is the duty of both central and reference laboratories to share the information they obtain with others.

Evaluations of methodology should be done in the setting in which they will be used. Careful planning of sampling, storage and transport to the laboratory is important. All samples should be evaluated by all assays in order to obtain a full view of value (Reid et al., 1995). The tendency today is to use a combination of tests for designating a true positive (Magnard et al., 1999). Before a single test can be considered a 'gold standard', methods must be standardized since reproducibility in different settings is important. Continuous internal quality assessment of test performance at all diagnostic levels is also a necessity.

In a recent comparative study, the sensitivity of different methods to detect influenza virus infection was evaluated for 154 specimens, 93 of which were positive by one or more methods: a PCR assay with the HA or M gene as a target, an ELISA, and cell culture (Magnard et al., 1999). The sensitivity varied with each method, but the most positive results were obtained with PCR using the HA gene as a target where sensitivity was 95%, whereas 43% were positive by ELISA and 83% by cell culture. No conclusions could be drawn about specificity.

Serology is usually not helpful for early diagnosis of respiratory virus infections, but with a sensitivity of around 80% it can be useful retrospectively for patient understanding and for epidemiologic studies. In general, ELISA assays perform better than hemagglutination-inhibition assays, which are better than complement fixation assays.

3.3. Detection of virus RNA using a real time PCR assay

Reverse transcriptase PCR provides relatively fast results (1–2 days), is sensitive (75–100%), and sensitivity can be increased by using a nested approach. However, the test is susceptible to contamination in routine laboratory settings, is technically demanding, and there are difficulties in implementation due to lack of standardization. The TaqMan® (Glaxo Wellcome) quantitative PCR is similar to an ordinary PCR in that specific forward and reverse primers are used; but, in addition, a probe is included that is labeled with a fluorescence reporter and quencher dye. As PCR progresses, the exonuclease activity of taq polymerase cleaves this probe and releases the fluorescent reporter which is then detected by laser based instrumentation. An important feature of this assay is that it is real time, as the fluorescence is being measured every 7 s, and a graphic of measurements is created over the time of the run. Thus, TaqMan® PCR offers a rapid result (usually 5–6 h), is sensitive, less prone to contamination as there is no post PCR sample handling, and is done with a standardized protocol.

The TaqMan® technique was used to detect influenza viruses. The HA gene segment was sought in a stock of A/PR/8 virus and the assay detected as far as 12 gene copies. Different vaccine and clinical strains of influenza were tested and all were detected with the same amplification efficiency. One patient was followed with serial sampling and a decreasing intensity of virus detected was observed with time.

In a pilot study, TaqMan® PCR was used to determine the viral load of different respiratory viruses in nasal swab samples obtained from patients with cystic fibrosis (CF). Viruses tested were rhinovirus (5′NCR), influenza (M1 protein–A/H1N1; A/H3N3; B), RSV (Polymerase), adenovirus (hexon), PIV 1 and 3 (HN), and coronavirus (NS1–229E and OC43). A nasal swab was obtained from 25 children attending a CF clinic and tested by blinded investigators using the TaqMan® PCR.

For RSV, a primer-probe was first designed and a region was amplified containing the amplicon of interest. The test was able to detect as little as one copy. In order to test variability of the test with swab samples, triplicate swabs were seeded with RSV, extracted and then tested in the TaqMan® assay. Results were consistent with a variability of around 15% (30% at very low concentrations). With one freeze–thaw, virtually all RSV was lost; influenza virus seemed to be less affected.

The TaqMan® method was then used to determine whether respiratory viruses can cause exacerbations in CF patients, to determine whether there is a threshold titer for this to occur, and whether there is a window of opportunity for therapeutic intervention. The threshold titers for clinical exacerbation appeared to be about 8000 gene copies. Viruses detected were influenza A, adenovirus, RSV, and rhinoviruses. The study suggested that it was feasible to determine viral load in respiratory secretions in CF patients using nasal swabs, and that it might be possible to correlate the viral load with clinical symptoms and determine a threshold titer for an exacerbation.

4. Pathogenesis and immunity

4.1. Pathogenesis of rhinovirus colds: implications for management

Rhinovirus (RV) enters the nose through direct contact or inhalation of infectious droplets that are rapidly (within 10–12 min) transported to the lymphoid area of the nasopharynx. Intracellular adhesion molecule-1 (ICAM-1), the receptor for most rhinoviruses, is present in abundance on the dome-like M cells in the epithelial layer. In studies done between 1960 and 1980, 95% of persons challenged with RV in their nose were shown to be infected (Gwaltney, 1997). The HID50 of RV is low (1–3 TCID50). However, only about 75% of those infected became ill. The most important risk factor for occurrence of a RV infection is the absence of specific antibody. The replicative cycle of RV in the nose is similar to that in cell culture systems, taking approximately 8–10 h and symptoms, such as 'scratchy' throat, appear in challenged individuals near the time complete virus is produced (8–12 h). The signs and symptoms of the common cold include rhinorrea, sneezing, sore throat, and cough. RV causes a significant rhinitis, but disease also involves the sinuses, causing a rhino-sinusitis, and, not infrequently, abnormalities in Eustachian tube function and middle ear pressure (Gwaltney and Ruckert, 1997).

The viral sinusitis is a self-limited illness characterized by thick secretions in the sinuses that are seen on computed tomography (CT) in up to 87% of adults. The maxillary sinus of an adult contains an average volume of 30 ml. Drainage occurs through the 3 mm diameter infundibulum. The sinus cavity contains a few seromucous glands, but many more goblet cells; RV infection may stimulate mucous production by these goblet cells. Also, nose blowing generates up to 60–80 mmHg of pressure while sneezing and coughing generates only about 8 mmHg of pressure. During nose blowing, radiographic dye placed in the nose is forced into the sinuses.

Symptom production is not directly attributable to viral replication. Multiple pathways of inflammation exist in nasal tissue, and specific symptoms are related in part to these pathways. Histamine stimulates sneezing, rhinorrea, nasal obstruction and sore throat. First generation but not second generation antihistamines block sneezing and reduce nasal secretion volume in patients with colds. Kinins cause vasodilation and increased vascular permeability resulting in increased nasal secretions, transudation of plasma, stimulation of pain nerve endings (sore throat), and stimulation of release of histamine by mast cells. Increased levels of kinins are observed in nasal secretions during both natural and experimental rhinovirus colds. Prostaglandins increase vascular permeability, induce bronchoconstriction, and, when given in the nose, result in rhinorrea, nasal obstruction and cough. Increased levels of IL-1, IL-6 and IL-8 have been found in secretions of patients with RV colds. The parasympathetic nervous system induces the sneezing reflex and secretion by mucous glands.

It is important to begin treatment with the first indication of a cold because most of the burden of symptoms occurs within the first 3 days of illness. Because nose blowing leads to deposition of nasal secretions within the nasal sinuses, early treatment may be useful. First generation antihistamines reduce the volume of nasal secretions, and the frequency of sneezing while nonsteroidal anti-inflammatory agents reduce cough, headache, and myalgia.

Approaches to prevention of rhinovirus infections are limited. A vaccine is not feasible because there are more than 100 serotypes. Blocking transmission is difficult in young children. Blocking viral attachment to the cellular receptor ICAM-1 has been only partially successful because of the difficulty of maintaining effective concentrations of soluble ICAM-1 in the nose. Stopping viral replication with drugs such as nucleic acid antagonists, capsid binding drugs and protease inhibitors, presents problems because of the difficulty of maintaining adequate nasal concentrations of the drugs, side effects and development of resistance. Also antiviral treatment alone has not been very effective in reducing illness. It appears necessary to also block the inflammatory pathways which are activated by the infection to achieve maximum symptom reduction.

There is evidence that combination therapy with antiviral and anti-inflammatory drugs leads to superior clinical efficacy. In a recent study using a RV challenge model with dosing beginning 24 h after virus challenge, the effect of administration of a combination of interferon in intranasal drops, clorpheniramine orally, and ibuprofen, was compared to placebo nasal drops and the two other drugs, and to placebo drops and capsules only. A sizable effect in reducing total symptom scores was noted in the full treatment group compared to the placebo. A trend for superior benefit from full versus partial treatment was also noted, suggesting that interferon provided an independent benefit.

In summary, RV reaches the nasal passages and is transported to the adenoids by mucocilliary action, it attaches to ICAM-1 receptors on the epithelial M cells and appear to first replicate in the adenoid area of the nasopharynx. Virus cytopathology activates inflammatory mediators and neurogenic pathways. These produce vascular transudation, pain nerve activation, and activation of sneezing and cough. Symptoms begin in the upper airway as early as 8–10 h after infection with abnormalities appearing in the osteomeatal complex, sinus cavities, Eustachian tube and middle ear, in 60–80% of individuals with RV colds. The symptoms begin to decline on the third day of infection and have a median duration of about one week. Viral excretion may continue for as long as 3 weeks, well after disease has subsided. The high susceptibility of the nose to infection, the rapid onset of replication, the early peak of viral shedding, the relatively brief opportunity for an antiviral treatment effect, and the role of inflammatory mediators in illness production support the idea of early treatment that attacks both the virus and mediators for achieving an optimal treatment effect.

4.2. The adenoviruses

The adenoviruses are common human pathogens that cause a broad spectrum of diseases, including conjunctivitis, keratoconjunctivitis, pneumonia, heart disease, hepatitis, nephritis, and gastroenteritis. They are ubiquitous viruses that typically exhibit latency and persistent shedding. Diagnosis is now enhanced by PCR, which allows specific typing; primers are available for all adenoviruses. There are 51 serotypes of adenovirus that are divided into six subgenera (A-F) that exhibit distinctly different organ tropisms. Additionally, recent molecular epidemiology has further divided many serotypes into genotypes designated a, b, c, etc. Different patient populations tend to acquire disease by specific adenovirus subgenra. Adenovirus subgenera A, B, and C are important causes of pneumonia in patients with primary immunodeficiencies. The B adenoviruses are predominantly found in renal transplant recipients, and C viruses in liver transplant recipients. Disease caused by certain adenovirus serotypes, particularly types 11, 31, and 35 is severe and often lethal in these populations. Some serotypes can cause severe and even fatal disease in immunocompetent children and adults. In healthy adults, severe lobar pneumonia has been associated with adenovirus 35; young children with adenovirus 3 pneumonia can develop bronchiectasis and hyperlucent lung; Ad1a has been associated with lung fibrosis; and fatal cases of Ad7 have occurred in children under 2 years of age.

In America, epidemic keratoconjunctivitis is caused by adenovirus 8. In 1955, adenovirus 19 was isolated in a survey for trachoma but it has not been associated with disease since then. Outbreaks of both genital and eye disease were caused by adenovirus 37. The only difference between the serotypes that caused keratoconjunctivitis and the ones that cause both genital and eye disease was found to be a single amino acid change.

Previously unrecognized disease entities have now been associated with adenovirus. In more than 300 children diagnosed with myocarditis, 19% were found to have adenovirus in biopsy material by PCR (Schowengerdt et al., 1996). Similarly, in 94 adult patients with left ventricular myocardial dysfunction but no myocarditis, adenovirus DNA was found in 12 (12.7%) (Paushinger et al., 1999) and all were adenovirus type 2. During an outbreak that occurred between April and June 1996, children died of a previously unrecognized entity characterized by cardiopul-

monary failure and arrythmias. A new virus called SIBU virus was identified in cerebrospinal fluid, brain or heart in 12 of 28 children (Cardosa et al., 1999). This virus was also associated with acute non fatal flacid paralysis in four children. This emerging virus is fastidious, shows tropism for the central nervous system and is associated with cardiac arrest; it appears to be related to subgenus B viruses.

Recent data have provided some understanding of the varying adenovirus tropisms. Virion fibers are of different lengths and serve as ligands between the capsid and host cell receptors. The variation in length is necessary for the virus to overcome differences in the charge of the viral capsid and the cell surface, which is usually acidic. Sequencing of the fiber genes of members of all subgenera revealed that the isoelectric point of the outer part of the fiber — the knob — ranged from 4.5 to 9.5. Within the fiber knob, two domains have been identified. The first domain contains tyr260 and arg279 which, when mutated to his260 and glu279, loses hemagglutination capacity. Adenoviruses with a predilection for the eye display a high positive charge of the fiber knob. For these adenoviruses the most alkaline region (with an isoelectric point of 10.4) corresponds to the second domain that is delimited by ala223 and lys251. These two amino acid residues are unique for adenoviruses that cause epidemic keratoconjunctivitis. Adenoviruses of subgenera A, C, D, E, and F exhibit affinity for the coxsackie–adenovirus host cell receptor (CAR), a pentameric integrin; but Ad37d, which causes epidemic keratoconjunctivitis, binds to sialic acid on host cell glycoproteins.

The pathogenicity of adenoviruses can be partly explained by the fact that they generally do not induce interferon production and are resistant to interferon action. In accordance with this, Ad41, which does not cause lethal disease, is sensitive to interferon. In this virus, sensitivity to interferon has been associated with the presence of a virus-associated RNA (VARNA) genome that is important for splicing. This RNA is transcribed by the virus' own RNA polymerase. If two VARNA genomes are found, the virus is resistant to interferon.

The importance of different adenovirus serotypes varies geographically. The molecular epidemiology of the respiratory adenoviruses has been studied since 1958. A mutually excluding switch from Ad7b to Ad7c took place in 1969 in Europe and in 1975 in Australia. In Beijing, Ad7d was predominant in 1980–1990, and has been succeeded by Ad7b. Adenovirus 7b is now the predominant pathogen in Beijing and the US, and Ad7c is predominant in Europe and Australia. Military recruits in the US received adenovirus vaccine that included Ad7a from 1971 until 1995. Since vaccination was discontinued there has been a re-emergence of cases. An outbreak of Ad7 occurred during the 1980s in South America causing unusually severe pneumonia and death. This emerging virus has been determined to be adenovirus 7h, a recombinant of containing an Ad3 fiber that has now appeared in Japan where Ad7 infections were formerly rare.

4.3. Respiratory viruses and otitis media

Acute otitis media (AOM) is the most common cause of physician office visits for children under 15 years of age and is the leading cause of antibiotic prescriptions in the US. The peak age for AOM is 6–24 months, and the highest incidence occurs during the winter virus season. Although the disease is generally considered a bacterial disease and treated with antibiotics, it is now clear that respiratory viruses play a significant role in both the pathogenesis, clinical course and outcome of AOM. Ten to 57% of children with a viral URTI will develop AOM concurrently or shortly after the URTI. The otitis media will develop within 4 days in 40–50% of cases, within 7 days in 60–75%, and within the first 2 weeks in 90–96%.

Viruses are detected in respiratory secretions and the middle ear fluid of a proportion of children with AOM (40–80 and 20%, respectively, depending on the diagnostic method, Chonmaitree and Heikkinen, 1997). The cause of the AOM is bacterial alone in 55% of cases, with *S. pneumoniae*, *H. influenzae* and *Moraxella catarrha*lis being the predominant pathogens. In 5% of cases, virus alone is found, and, in 15% of

cases, a combination of virus and bacteria is detected (data compiled from UTMB studies 1982–1998). With current diagnostic methods, no pathogen is found in about 25% of the cases. Respiratory viruses that are associated with AOM include: RSV, parainfluenza viruses, influenza viruses, rhinovirus, enterovirus, adenovirus, coronavirus, cytomegalovirus and herpes simplex virus. In a study by Heikkinen et al. (1999), RSV was shown to be the most ototropic virus, with a middle ear invasion in 74% of children with AOM who were infected with RSV. The rate was 52% for parainfluenza, 42% for influenza, 11% for enterovirus and 4% for adenovirus. Also, influenza vaccination in children has resulted in significant reductions in AOM during influenza season (Belshe et al., 1998).

Data from in vitro studies, experimental animals, adult volunteers, and children with URI and AOM suggest that viruses may be involved in the pathogenesis of AOM in several ways. Infection of the upper respiratory tract induces production of various cytokines and inflammatory mediators such as histamine, kinins, interleukins, arachidonic acid metabolites, TNF-alpha and possibly other mediators. Many of these substances have been shown to induce an inflammatory process that leads to mucocilliary damage, dysfunction, and impaired middle ear ventilation. Experimental influenza infection in animals results in middle ear pressure changes, tympanic membrane inflammation, damage, disappearance of ciliated epithelial cells, and increased Eustachian tube mucous and cellular debris. In preschool children with URI, a significant negative middle ear pressure occurs by the second day of illness. Eustachian tube dysfunction also occurs in adults during rhinovirus and influenza A experimental infection.

Viruses may also alter neutrophil function and enhance nasopharyngeal bacterial colonization and adherence. These events along with Eustachian tube dysfunction then lead to invasion of the middle ear by bacteria and virus. Once the microbial agents reach the middle ear, another inflammatory cascade is initiated and this leads to middle ear fluid accumulation and symptoms of AOM.

Evidence to date also suggests that active viral infection of the middle ear or the respiratory tract at the time of AOM diagnosis is associated with a poor response to antibiotics. Effective use of viral vaccines to prevent respiratory viral infections or effective treatment with antiviral drugs will likely lead to reductions of the incidence and improvement of outcomes of this very common disease.

4.4. Immune correlates in RSV disease

The F and G surface glycoprotein of RSV are involved in the host immune response, particularly antibody responses. Most of the other viral proteins are involved in T cell responses. Young children under 6 months of age will often have severe RSV bronchiolitis, and repeated infections may occur. Factors contributing to the severity of disease at a younger age include the immaturity of the immune system and the presence of maternal antibodies transferred through the placenta and collostrum, which may interfere with immune responses until they decline naturally. However, passive administration of antibodies has been shown to confer protection against RSV in young infants.

A controversial area in the pathogenesis of RSV is the role of T cell induced immunity. Based on observations in the 1960s with a formalin inactivated vaccine used in infants, and from experiments in animal models, it has been speculated that an enhanced pulmonary histopathology was related to a higher CD4 T-cell response to infection accompanied by a shift from a Th1-like response towards a Th2-like response. Both CD4 and CD8 T cells are involved in recovery and pathogenesis of RSV disease, and the balance between protective and disease-enhancing effects of T cells is important in considering development of intervention strategies. It has also been observed that there is a correlation between severity of disease and increased IgE and histamine production, especially in children with asthma. Eosinophils which are recruited and which degranulate in the lung parenchyma may contribute to the lung pathology. For the development of prevention strategies, it is important to induce both mucosal and systemic antibodies and to

understand the mechanisms for pathogenesis of RSV disease.

The identification of T-cell epitopes in RSV and their role in immunopathogenesis and as vaccine targets is important. T cells from two infants <2 months of age with severe RSV disease were cloned by limiting dilutions and screened for RSV specific proliferative responses. The clones obtained in this way were mostly $CD3^+$ and $CD8^+$ and each clone produced mainly interferon gamma, a Th1 phenotype marker. From one of the children, specificities for the F protein and the 1C protein were found; in the other, the major specificity was for RSV itself but some clones were reactive with the F protein and the 1B protein. Little or no reactivity for the G or other proteins was found. All the clones specific for the F protein reacted with one of two peptides. The MHC restriction of the recognition was tested by using panels of B cell lines; cells that reacted were B57 or C12. Thus, these experiments describe the identification of two epitopes on the F protein with specific genotype restrictions.

To understand the pathogenesis of RSV in very young children, a cohort of 120 infants with acute respiratory infection within the first 6 months of life was followed. Those with severe and mild infection were separated into comparison groups. Cytokine measurements 3–4 weeks after infection revealed a difference only in the increased production of IL-6 and IL-8 pro-inflammatory cytokines in the plasma of those with more severe disease. The inflammatory cells in nasopharyngeal washings during the infection were analyzed for CD4, CD8, and CD3 lymphocytes, CD15 polymorphonuclear cells, IgE, eosinophils, and mast cells. Little or no difference was observed in children with mild versus severe RSV disease. Again, there was no indication of a Th2 response. RSV specific T cell responses were sought during the acute (admission) and convalescent (3–4 weeks after) phases of infection in these children. In the acute phase, there was no indication of selective IL-4 or IL-10 production in the severe group when compared to the mild group. In the convalescent phase, there were no differences between the two groups in the induction of the various cytokines. Intracellular staining was performed for interferon gamma and IL-4 in CD4 and CD8 cells, and, again, no clear differences were observed between the severe and the mild group.

In conclusion, infants with more severe RSV infection exhibit higher levels of plasma IL-6 and IL-8, but a more pronounced RSV specific type 2 like response was not observed. Few eosinophils were found in NP wash specimens. The predominant cytokine produced was interferon gamma, and only minimal amounts of IL-4 or IL-10 were noted. These data do not support the hypothesis that severe RSV infection results from a Th2 lymphocyte response.

4.5. HLA restricted CD8 + CTL, IFN-γ and IL-4 responses to RSV infection in infants and children

Cell mediated immunity (CMI) is thought to play a role in clearing viral infections. Normal children may shed RSV for up to 21 days, while CMI deficient children shed virus for months. Moreover, adoptively transferred T-cells will clear viruses in immunodeficient animals. There are a number of studies in animals and in adults and children showing that primary infection with RSV induces Th1 responses predominantly; cytotoxic lymphocyte (CTL) responses are a Th1 response. In a study of normal children 6–24 months of age with RSV infection, CTL responses were not correlated with disease severity. But, in another study, CTL responses were detected in 4/22 infants hospitalized with bronchiolitis, and those with CTL had mild disease. To evaluate and compare RSV specific CTL responses and cytokine production in infants and children, and to identify their role in immunity and disease with RSV, infants were enrolled at birth and followed for three consecutive years. Blood was obtained before and after each RSV season as well as during any acute respiratory infection that required medical attention.

In year one of study, 18 of 26 infants were infected, 12 were detected by virus isolation, and six by antibody rise. By year 3, only one child had not had infection. A standardized CD8 CTL assay was used. The average CTL activity for the 26 infants was low at enrollment and progressively

increased during the first season. This increase of CTL activity was related to exposure to RSV. A low level of interferon gamma in assay supernatants was noted at enrollment and the level also increased with time. IL-4 was quite high initially, and as the season progressed, the average level decreased. A direct correlation was observed between CTL activity and interferon gamma level while an inverse correlation was observed for IL-4. Infants who had virus isolation were among those with medically attended lower respiratory disease; they tended to develop CTL activity. Those who developed CTL activity in year 1 had a reduced likelihood of a medically attended illness in year 2 even if they were infected.

5. Vaccines

5.1. Current status of intranasal live attenuated influenza vaccine

Significant advances in the use of live attenuated cold adapted influenza vaccines include the following: the vaccine is updated annually to match the FDA recommendations for the trivalent inactivated vaccine; it is produced consistently to a titer of $10^{6.7}$–$10^{7.0}$ TCID 50 per strain; a nasal spray device that produces a large particle aerosol is used to deliver the virus to children and adults; it takes approximately 10^4 pfu of each strain to infect 50% of seronegative children; virus is shed in respiratory secretions with a titer of 10^3/ml for about 10 days in seronegative children (reversion to wild type has not been detected); monovalent, bivalent and trivalent vaccines are well tolerated up to a dose of 10^7 pfu per strain per dose; some interference is seen between the strains, but this can be overcome by administration of a second dose a month later; and a broad antibody response is observed, including serum and secretory IgA antibody.

Clinical trials with the cold-adapted, attenuated influenza vaccine in children and adults have indicated that it is suitable for general use to prevent influenza. In a two year multicenter, double-blind, placebo-controlled, efficacy trial of the vaccine by nasal spray to children 15–71 months of age, overall vaccine efficacy was 92% for preventing culture confirmed influenza A/H3N2 (A/Wuhan) and influenza B (Belshe et al., 1998). In the second year of study, vaccine was 86% effective at preventing influenza from A/Sydney virus, a drifted H3N2 variant. Natural A/Wuhan infection in year 1 in placebo recipients led to 85% efficacy against A/Sydney in year 2. The live vaccine, therefore, appeared as effective as natural infection in this instance. Children under 2 years of age, for the most part seronegative, had a significantly higher response to the vaccine than older children. However, there was no difference in the protective efficacy of the vaccine among different age groups. The vaccine was also highly protective against influenza associated otitis media. Furthermore, influenza illness in vaccinated children was milder than in placebo recipients and lower respiratory disease occurred in the placebo recipients only in year 2.

There were no serious adverse events associated with the vaccine in this trial. On day 2 after the first dose, there was a significant increase in rhinorrea, and a small increase in the incidence of low grade fever in vaccine recipients. This was not observed after dose 2 or after revaccination 1 year later. Otitis media was not increased in the 10 days post vaccination comparing vaccine to placebo groups and was not higher than the background rate. Among subjects who came from a household with two or more siblings, seroconversion to the vaccine virus was observed only in vaccinated participants, suggesting that the vaccine virus did not transmit.

Although the endpoint of the pediatric trial was not to determine the effectiveness of the vaccine, the following observations were made: there was a 21% reduction of febrile illness in year 1 and 19% in year 2 among vaccinees; there was a 9 and 8% reduction in all cases of otitis media in year 1 and 2, respectively while febrile otitis media was reduced by 33% in year 1 and by 16% in year 2; a significant reduction was observed in antibiotic use, days of missed daycare for children, and in work lost for parents.

Since influenza A/H1N1 did not cause disease during the years in which the study was conducted, determination of efficacy against experi-

mental challenge with the attenuated H1N1 vaccine strain by intranasal spray was performed. Vaccine virus challenge was not associated with serious adverse events. Vaccine was 83% efficacious (CI = 60–93) at preventing shedding of H1N1 virus after challenge. Any serum HAI antibody or any nasal wash IgA antibody in prechallenge specimens was correlated with significant protection from H1N1 infection. This study duplicates the pattern of protection that was observed during natural influenza outbreaks of influenza A/H3N2 and influenza B in children. Furthermore, it indicated that children who were protected against viral shedding had some measurement of immunity, either mucosal IgA, or serum HAI or neutralizing antibody.

Effectiveness trials in working adults demonstrated that influenza-like illness was significantly reduced (21%) by intranasal live attenuated vaccine during the 1997–1998 influenza season (Nichol et al., 1999). Vaccine reduced lost work days by 22% and reduced antibiotic and over-the-counter medication use by 32% each. Clinical trials in the elderly, in subjects with COPD, in HIV-I infected adults and in patients with asthma have revealed the vaccine to be safe. Use of live attenuated vaccine in the elderly as a combined vaccine with the inactivated vaccine is currently under evaluation.

Thus, intranasal live attenuated vaccine is a significant new tool to prevent influenza and its complications. General use of the vaccine in healthy children and adults age 1–65 is supported by the clinical trial data. Use in special populations and in the elderly is under study. Comparative studies of live attenuated vaccine versus inactivated vaccine are necessary. However, current trials indicate that live attenuated influenza virus vaccines might result in a broader immune response among young children (Belshe et al., 2000a,b).

5.2. Live cold adapted influenza vaccine in the community

Mortality associated with lower respiratory tract disease has shown a substantial and steady increase since 1982. Excess mortality from influenza also appears to be increasing (Simonsen et al., 1997). From 1974 to 1984 the annual average number of deaths from influenza was 15 500, increasing to 30 000 from 1984 to 1992. Accompanying the increase in mortality, has been a 50% increase in the rate of hospitalization for pneumonia in people over 65 years (1985–1995). Strategies for prevention of LRTI are needed.

The purpose of a study being performed in Temple, TX, is to determine the indirect effects of universal vaccination of young children on influenza epidemics in a community. Specifically, does immunization of pre-school and school aged children with live attenuated influenza vaccine significantly reduce medically attended illnesses in community contacts regardless of their immunization status. The vaccine is easy to administer and is well tolerated by children; it has the potential to be used for this purpose in any community.

The study community is a large clinic in central Texas. Data for comparison are being obtained from clinics and an HMO in three other central Texas cities. Data for 1998–1999 is available for estimating vaccine efficacy in the study population; 4298 children were vaccinated, approximately 24% were 18 months–4 years of age, 40% were 5–9-years-old, and 36% were 10–18 years of age. No serious adverse events or serious illnesses sometimes associated with influenza occurred during the 6 weeks immediately after vaccination. Medically attended acute respiratory illness (MAARI) rates were not increased following vaccination when compared to pre-vaccination or >15 days post-vaccination.

Virus cultures of persons seeking medical care for an acute respiratory illness was used to define the beginning and end of the influenza season. Influenza B was isolated from 45% of persons with culture positive influenza in the study and comparison communities; co-circulation of RSV occurred during the epidemic. A total of 4298 children were vaccinated. Direct effectiveness of the vaccine was demonstrated during the most intense period of the influenza season. For the combined age groups a significant reduction in MAARI was observed (18%). For the 18 months–4 years age group, reduction was 21% when compared to age matched eligible non vac-

cinees in the community. In the 5–9 year age group, a 13% reduction was noted; and in the 10–18 years of age group, the reduction in MAARI was 34%.

5.3. Neuraminidase based vaccines

That the influenza virus surface glycoprotein antigen, neuraminidase (NA), has a role in immunity has been recognized for years, yet it remains an unmonitored component of inactivated vaccines, and its potential for engendering infection-permissive immunity when administered as a single vaccine component has not been exploited. The studies reported here are concerned with efforts to improve present vaccines by supplementation of the predominant hemagglutinin (HA) antigens with recombinant NA (rNA) (Couch et al., 1974; Johansson et al., 1995, 1998). Based on extensive animal studies and previous clinical trials with HA/NA antigenic hybrids, we anticipate that addition of purified NA will: (1) insure that the NA in vaccines is present in adequate amounts; (2) balance the ratio of HA to NA to reduce antigenic competition in which the predominant anti-HA response is suppressive to the anti-NA response; (3) provide adequate amounts of an antigen less subject to antigenic drift (NA), thus providing back-up immunity when the vaccine HA does not match well that of a new virus.

In mice, rNA elicits protective NA antibodies effective against homologous challenge with A/Johannesburg/33/94 (H3N2) virus (more than 200-fold reduction in lung virus), and also reduces lung virus titers by almost 100-fold following heterovariant A/Nanchang/933/95 (H3N2) virus challenge. After measuring the dose response to baculovirus recombinant (rNA Johannesburg) in humans in Cincinnati, and verifying its safety and immunogenicity in doses ranging from 2.5 to 45 μg, we carried out a phase 2 clinical trial in a similar Cincinnati population. In this trial, groups of 20–25 subjects received either TIV (a commercial subvirion trivalent vaccine, Fluzone™), TIV + rNA, or placebo. As in the mouse model, rNA was derived from A/Johannesburg/94, the same H3N2 virus present in the 1995–1996 TIV vaccine. Subjects were challenged with A/Nanchang/95 virus, the succeeding heterovariant virus in which we have shown the HA to be 43% and the NA 78% antigenically related to that of the vaccine virus.

In summary, we demonstrated that, in comparison with TIV alone, rNA + TIV results in an increase in NA antibody titers, and is associated with less viral shedding, and with a reduction of symptomatology, all in the face of non-homologous challenge. rNA, as the first recombinant NA used in humans, is safe and immunogenic alone or in combination with TIV.

Difficulties confounding this evaluation include: (1) a closer than anticipated antigenic relationship between the HA's of A/Johannesburg/94 and A/Nanchang/95, so that immunity to A/Johannesburg/94 resulted in reduction in infection in both rNA + TIV and TIV groups; (2) the A/Nanchang virus challenge was attenuated to the extent that only mild URI symptoms occurred in the placebo group; and (3) inadequate numbers of volunteers to show statistical significance although most data on viral shedding showed uniform trends in favor of supplemented vaccine.

Our background studies as well as data presented indicate the importance of correction of the probable NA deficiency in present vaccines. Adequate levels of NA antibody may reduce viral shedding and therefore chances of disease transmission. We have shown a more balanced response to HA and the more slowly evolving NA antigen which offers a back up against HA drift.

5.4. Evaluation of two doses of either subunit or MF-59 adjuvanted subunit influenza A/duck/Singapore-Q/F119-3/97 (H5N3) vaccine in healthy adults

As part of preparation for a future influenza pandemic, there is a need to evaluate conventionally-prepared and novel influenza vaccines in immunologically naïve populations. In a phase I study in 66 healthy adults, A/duck/Singapore-Q/F119-3/97 (H5N3) was given as 7.5, 15 and 30 μg of the H5 HA on days 0 and 21 in an observer blind, randomized, parallel dose ranging study.

There were no serious adverse events, although there was a greater incidence of local pain in

recipients of the MF-59 (an oil-in-water adjuvant) adjuvanted vaccine (Ott et al., 1995). Serum H5 HI antibody titers $\geq$1:40 developed in 0/32 and 4/32 subjects 21 days after the first dose, and in 1/32 and 12/31 subjects 21 days after the second dose of conventional and adjuvanted vaccine, respectively. A trend towards an increasing response with increasing doses was noted with the conventional vaccine, but the opposite was true for the adjuvanted vaccine. Increases in neutralizing antibody titer to $\geq$1:20 developed in 4/32 and 12/32 subjects 21 days after the first dose, and in 7/32 and 29/31 subjects 21 days after the second dose of conventional and adjuvanted vaccines, respectively, and there was a trend for an increased response after receipt of a second dose of adjuvanted vaccine. The geometric mean titer (GMT) of both HI and neutralizing antibodies on days 21 and 42 were not significantly different from prevaccination levels in conventional vaccine recipients, but each was significantly greater for adjuvanted vaccine.

The response elicited by the conventional vaccine suggests that it cannot be relied upon to evoke protective antibody levels in humans against an emerging pandemic influenza A virus. The MF-59 vaccine clearly boosted the HI and neutralizing antibody responses in immunologically naïve subjects. At least two doses of vaccine appear to be required to elicit an adequate response.

5.5. Cross reactive immunization against influenza A viruses: T lymphocyte approach to improve influenza vaccine for the next pandemic

T cells see processed viral peptides on the surface of infected cells that are presented in conjunction with histocompatibility molecules. Animal studies have demonstrated that passive transfer of CD8 positive T cell clones possessing specific lymphocyte cytotoxic capability for influenza virus will reduce pulmonary virus loads and protect mice against death. However, the studies in mice raise concerns about the utility of this approach because, in general, the T cell repertoire in mice is very limited. At present, there is very limited data in humans; only a handful of epitopes for influenza A have been identified in non-systematic studies of human lymphocytes.

In a recent series of experiments, human lymphocytes were stimulated with PR/8 virus. In bulk culture cytotoxicity tests, there was killing against H1, H2 and H3 viruses to a high degree. Using recombinant vaccinia targets, a heterogeneous CTL response was noted with NP being highest, but half a dozen other proteins were recognized. Lines of the PR/8 stimulated culture were cloned, and the histocompatibility proteins involved in recognition by one donor were determined. HLA B27 was able to present multiple epitopes on NP, as well as epitopes on PB1, PB2, NS1 and NS2 to CD8 CTLs. A smaller number of CD4 clones were detected, and they mostly reacted with epitopes on the HA and NA.

After emergence of the avian influenza A H5N1 virus in Hong Kong in 1997, the clones of human lymphocytes were tested against this and other non human virus species. Most of the clones tested were able to kill swine A/New Jersey (H1N1) virus expressing targets, and both of the Hong Kong strains of H5N1, as well as the control PR/8 virus. Therefore, the human CTL response to influenza A virus is largely cross reactive, as many of these cross reactive CTLs recognize conserved epitopes on avian and swine influenza A viruses as well as those on human viruses.

CTL responses in subjects who received a standard inactivated influenza vaccine or vaccine formulated with immunostimulating complexes (iscoms) were quantitated. The influenza vaccines formulated with an iscom carrier were shown to be taken up by human cells and to stimulate influenza A virus specific CD8+ T cells in vitro. A double blind clinical trial demonstrated that, after one dose, the iscom vaccine significantly increased the level of influenza A virus specific cross reactive CD8+ T cell memory in about 60% of recipients as compared to 5% in the standard vaccine group. The levels of increase in CTL memory cell responses correlated with increases in the levels of IFN gamma producing T cells. It is proposed that augmentation of these CTL memory T cells should be a goal in the design of influenza A vaccines against emerging influenza A viruses.

5.6. Attenuation of influenza virus via genetic engineering

Reverse genetics of influenza virus was initiated several years ago. Ribonucleoprotein complexes (RNPs) were reconstituted in vitro from RNA transcribed from plasmid DNA in the presence of polymerase (Pol) proteins and nucleoprotein isolated from purified influenza virus. The in vitro-reconstituted RNPs were transfected into cells that were also infected with a helper influenza virus; helper virus provided the remaining viral proteins and RNA segments, resulting in the generation of transfectant viruses.

Helper virus-based rescue systems using a RNA Pol I promoter-driven reverse genetics technique were subsequently established for the segments encoding the neuraminidase (NA), the hemagglutinin (HA), the NS1 and nuclear export proteins (NS2), the M1 and M2 proteins, the nucleoprotein (NP), and the polymerase 2 basic protein (PB2). These results then led to the development of an entirely plasmid driven system where a mixture of eight plasmids expressing individual RNA segments of influenza virus from a truncated human Pol I promoter was used to recover infectious virus from cloned cDNA. In addition, the previously used RNA complex plasmids were replaced with plasmids expressing the same proteins under control of the adenovirus 2 major late promoter. Using this system, 10^4–10^5 infectious particles can be obtained in 48 h in a Vero cell culture (Fodor et al., 1999).

5.7. Influenza: role of nonstructural (NS) protein

The NS gene codes for the NS1 protein and, from a spliced messenger RNA, for the NS2 protein. Many different functions have been described for the NS1 protein but perhaps the most important activity in the host cell is that of interferon antagonism. Influenza A and B virus expressing altered NS1 proteins result in altered interferon responses, and these mutants represent a potential novel vaccine (Garcia-Sastre et al., 1998; Talon et al., 2000).

When PR/8 virus is used to infect the cell, the cell produces interferon, but its activity is countered by a strong anti-interferon response of the virus, so the virus is able to productively infect the cell. A genetically altered virus (NS1 99) encodes a full length NS2 but only the amino terminal 99 amino acids of NS1. Another genetically derived virus (Delta NS1) lacks an open reading frame for NS1. The Delta NS1 virus infects the cell and stimulates it to produce interferon, but the virus does not induce an anti-interferon activity so its replication is restricted by the host. The NS1 99 virus is intermediate in effects and is attenuated in vitro.

Embryonated eggs of different ages have a different capability for inducing interferon. The three viruses were inoculated into different aged embryonated eggs. A titer of 10^8 PFU was obtained for PR/8 in eggs 6–10-days-old. The Delta NS1 grew up to 10^7 in eggs up to 6-days-old but more than a $\log_{10}$ reduction was observed in 8-day-old eggs, and there was no growth in 10-day-old eggs. The NS1 99 virus showed intermediate growth between that for the PR/8 and delta NS-1 viruses. When mice were infected with the viruses, PR/8 had an LD50 of 10^3 PFU. The delta NS1 did not kill normal mice while the NS1 99 virus has an LD50 of about 10^6. When mice were immunized with a single dose of 10^6 PFU of Delta NS1, complete protection was observed, but a lower dose of 10^4 did not protect. The NS1 99 virus at either dose protected most or all immunized mice when challenged 4 weeks later with either 100 or 5000 LD50.

In the influenza B virus system, the NS gene also codes for the NS1 protein and the nuclear export protein (NS2). Two viruses, B/201 and B/234, containing truncated NS1 proteins, 224 and 90 amino acids, respectively, were derived from B/Yamagata virus. As for influenza A, the virus with the shorter NS1 would not grow in older embryonated eggs while wild type virus grew at high titer in 10-day-old eggs. The B/201 only grew in eggs 6–8-days-old and the B/234 in 6 day-old eggs only. Since B/Yamagata wild-type did not result in lethality of infected mice, lung titers were measured after challenge. A titer of 10^4 PFU/g was observed in the lung after wild virus challenge while the two attenuated strains had barely detectable levels. After 4 weeks, the mice

infected with either B/201 or B/234 were completely protected against a wild type challenge.

In summary, influenza virus infecting a cell will induce an interferon response, and the cell will win unless the virus has strong anti-interferon activity mediated through the NS1. Attenuated strains of viruses can be produced by preparing NS1-based intermediately attenuated strains that are neither temperature sensitive nor cold adapted. The principle of modulating the anti-interferon response of a virus for vaccine development may not only work for influenza virus but also for other viruses.

5.8. Attenuated vaccines: preclinical aspects

The molecular biological technique of reverse genetics is being utilized to develop live attenuated virus vaccines for subgroup A and B strains of RSV and for parainfluenza viruses types 1, 2 and 3 (PIV1-3) to prevent the serious lower respiratory tract disease caused by these viruses. The genetic basis of attenuation has been defined for various RSV or PIV vaccine candidates and this information has been used to generate a large menu of attenuating mutations for both RSV and PIV. Specific mutations such as deletions and animal gene substitutions offer the possibility of heightened stability of the attenuation phenotype following replication in vivo. It is now possible to combine mutations from this menu to derive novel attenuated viruses that exhibit the proper balance between replication, attenuation, and immunogenicity that is needed for a successful live attenuated vaccine. It is also possible to rapidly produce new vaccine candidates by antigenic chimerization in which the protective antigens of one virus are substituted for those in a serotypically different vaccine candidate that has previously been shown to exhibit the right level of attenuation. These attenuated RSV and PIV viruses can be used as vectors for the protective antigens of other human viral pathogens.

5.9. Clinical status of paramyxovirus vaccines in children

RSV and PIV strains are currently being evaluated in children in phase I, blinded, randomized trials. Generally the RSV vaccine is given as nose drops in one dose of 10^4–10^5 PFU but two doses at a 1 month interval have been given to younger children (Karron et al., 1997).

Infection with an RSV cold adapted-temperature sensitive attenuated vaccine (cpts 248/404) did not result in febrile or lower respiratory tract disease in seropositive and seronegative children 6–24 months of age, nor in children 1–2 months of age. In the younger children nasal congestion occurred about 8–10 days after vaccine administration, lasting 24 h and it was accompanied by difficulty sleeping or feeding. This, along with the fact that the virus was replicating at fairly high titers, indicates a need for further attenuation of this virus. There was no relationship between the amount and pattern of virus shedding in the upper respiratory tract and the level of maternally acquired antibody or age of the child. In the youngest children, minimal virus shedding was noted after a second dose of vaccine among those who shed virus after the first dose. The protection conferred, however, was not apparently related to the development of a detectable serum IgG antibody response. Most children over 6 months of age had a serum IgG antibody response to the F and/or G protein in ELISA; however, only a few children under 6 months of age demonstrated a response after two doses, and virtually none after a single dose. However, 80% of 1–2-month-old infants developed serum IgA antibody and 40% developed nasal wash IgA antibody. Antibody was mostly directed to the G protein. Viral shedding was noted after the second dose of vaccine only among those with no serum IgA antibody response. Thus, a potential correlate of immunity was identified. In the 6–24 month old children, protection against RSV associated URTI and otitis media was observed in vaccinated children in the subsequent season.

Live attenuated PIV vaccines have also been evaluated with an aim of induction of serum and mucosal antibodies (Karron et al., 1995a,b). A PIV3 vaccine passaged 45 times at 20–22°C

(cp45) is cold adapted, temperature sensitive, and attenuated in a variety of animal models. A second vaccine candidate, which was derived from a wild type bovine PIV3 virus, was similarly attenuated in humans. Doses of 10^5–10^6 of either PIV vaccine were administered in nasal drops to older children; in younger seronegative children dose ranges of 10^2–10^5 were tested. Neither of these vaccines induced respiratory illness. The viruses are poorly infectious in seropositive children. In the 6–36 month old group, 81% had an antibody rise after the cp45 vaccine, 85% after a bovine vaccine with surface antigens which share homologies with human PIV3.

Therefore, in older seropositive children and adults, vaccines like cp45 and the bovine strains are safe and immunogenic. When given to 6–36-month-old seronegative children, a high level of infection is seen as judged by antibody responses and virus shedding; the virus shed has been shown to maintain its ts and cold-adapted phenotypes. A lower dose of vaccine reduces the infection rate but not the amount of shedding.

Trials have now been started with the cp45 human PIV 3 vaccine in 1–3-month-old children given a two dose regimen at 1–3 month intervals. When given 1 month apart, the vaccine is attenuated, is genetically stable, and retains the ca phenotype. With the first dose of vaccine all children shed virus for a period of about 15, days and the mean peak titer is around 10^3 TCID 50/ml of nasal wash. Only 24% of children shed virus after the second dose of vaccine and the shedding period was shorter and the mean peak titer lower. No serum HAI responses occurred after either dose but, as with RSV, a serum IgA response was seen.

Overall, these parainfluenza vaccines are safe for healthy children as young as 1 month of age; they replicate efficiently in these young children, are genetically stable, and induce serum IgA but not detectable neutralizing or IgG antibody responses. The major component of inhibition of serum antibody production from these vaccines in very young infants seems to be the level of maternal antibody. As with RSV, a single dose protects against a vaccine challenge.

5.10. Safety and immunogenicity of a recombinant subunit RSV vaccine

As an alternative to a live attenuated RSV vaccine, a novel recombinant RSV subunit vaccine using a fragment of the G protein, designated BBG2Na, is under investigation. The vaccine is composed of a fragment of the streptococcal protein G albumin binding domain fused genetically to G2Na, a highly conserved 130–230 amino acid fragment of the RSV G protein. This vaccine is highly purified and well characterized. It is immunogenic and protective in rodents and non-human primates as well as efficacious in a neonatal murine model in the presence of high levels of maternally derived antibodies (Power et al., 1997; Siegrist et al., 1999). There has been no evidence of enhanced pathology with challenge of animals given vaccine.

The objective of the study was to determine the safety and immunogenicity of BBG2Na in healthy seropositive young adults. Subjects received 10, 100 or 300 μg of BBG2Na in aluminum phosphate ($n = 81$) or saline placebo ($n = 27$), once, twice or three times at monthly intervals. Local reactions consisted of mild pain at the injection site in 27/81 vaccine recipients compared to 1/27 placebo recipients. There were no systemic reactions or serious adverse events from vaccine. Antibody responses in ELISA and neutralization assays were higher in the 100 and 300 μg groups after both 1 and 2 injections. Twofold rises in RSV-A ELISA antibodies were evident in 100 and 89% of subjects in the 100 and 300 μg groups, while fourfold rises of antibody were seen in 57 and 67% of subjects, respectively. Up to 75% of subjects demonstrated a twofold increase in the level of neutralizing activity. There was a tendency for a second immunization to boost the neutralizing antibody response but there was no benefit from a third dose. The level of antibody response was inversely related to the pre-vaccination titers. Thus, BBG2Na appeared to be safe and immunogenic in RSV seropositive adults following intramuscular administration.

6. Antivirals and other treatments

6.1. Neuraminidase inhibitors: rational drug design

The structure of neuraminidase was discovered in 1983 (Colman, 1994). The catalytic site of this enzyme is strain invariant and inhibitors of the enzyme will have a broad spectrum of activity against influenza viruses. The product of the enzyme reaction, sialic acid, sits in the enzyme catalytic site with a high degree of chemical and topological fit. An exception occurs where the 4 hydroxyl of the sugar is inserted into a H_2O filled cavity of the protein, the surface of which is lined by two carboxylate groups from two glutamate amino acids. The development of anti-neuraminidase agents was driven by this observation and the consideration that building more basic and bulky substitutes for the hydroxyl group might improve fit into the site. The first two serious candidates in the late 1980s were 4-amino and 4-guanidino substituted dehydro-sialic acids, the latter known as zanamavir (Varghese et al., 1998).

Small changes in the molecule resulted in dramatic improvements in potency. Replacing the hydroxyl with an amino group and a transition from ammonium to guanidinium improved the KI by a factor of approximately 100 in each case. Moreover, this substitution made the compound more soluble. Reducing the solubility of the protein increases the binding constant, and permits delivery in ways other than topical. This was attempted by replacing the glycerol side chain of the sugar with carboxamide linkages, and attaching hydrophobic substituents, such as a methylpropyl or diethyl side chain. However, these substitutions did not result in dramatic changes in the distribution properties of zanamavir. The alternative compound (oseltamivir) is also a diethyl substitution at the glycerol position of the C6 analogue of pyranose. These compounds bind satisfactorily into the active site of the neuraminidase because the hydrophilic pocket that accommodates the glycerol already has a hydrophobic component to it; a hydrophobic surface is exposed down the hydocarbon backbone of the guanidinium which the back side of the glycerol interacts with. The hydrophobic accommodation of the carboxamides is made complete by also creating a hydrophobic environment in its vicinity.

A number of resistant variants of influenza have been selected with zanamavir and oseltamvir. Some of those variants are in the HA. The HA is not the drug target, but the mutations arise because of the balance that needs to be maintained between binding and receptor destruction in the virus cycle. Active site variants of the NA have been seen now in a number of sites in the enzyme active site. Glutamate 119 and 227 are the sites that directed the design of zanamavir and glutamate 119 has now been seen to mutate in resistant variants to three different amino acids, glycine, alanine and aspartate. An arginine 292 to lysine mutation has been selected with zanamavir or oseltamivir in the laboratory and with oseltamivir in clinical studies. A single reported case has been reported of a mutation of arginine 152 that resulted in resistance in an immunocompromised child receiving long term therapy with zanamavir.

Most of the NA mutations result only in local effects. In the case of Arg292 to lysine, by virtue of the charge not being projected as far into the active site pocket, it ends up in a salt link with glutamate 276, a residue important for creating the hydrophobic pocket for glycerol substituted compounds. Resistance index measurements show that progression further and further from the natural substrate results in more dramatic consequences of the arginine 292 to lysine mutation. The four amino compound bound identically to wild type NA and to the arginine 292 variant and has a resistance index of 20 or 30. Oseltamivir with a resistance index of 6500 does not enter the pocket. The likelihood of generating that situation increases as the drug becomes less similar to substrate. A drug resistance variant is one that maintains its ability to process natural substrates but is increasingly able to discriminate between the drug and substrate.

Zanamavir is less similar to the natural substrate in the part of the pocket where glutamate goes to glycine, than oseltamivir. Therefore, in

relation to the glutamate 119 to glycine substitution, the resistance index is higher for zanamavir than it is for oseltamivir. One of the encouraging factors for oseltamivir is that this resistance factor is never more than 10–100-fold. The importance of the resistance index in practice is to determine that there is sufficient drug at the site of infection to deal with breakthrough strains.

Designing ligands to nanomolar potency in an optimized mode is now becoming routine. Quantifying and even ranking the binding potency of a range of ligands by computation, however, remains very difficult; whereas, designing for pharmacological features seems to be increasingly possible. Structural considerations can direct where to add desirable groups without altering the molecule's binding properties. Many more drugs should be discovered using these methods in the future.

6.2. Update on resistance to neuraminidase inhibitors

Because of the interaction between NA and HA, it is possible to derive neuraminidase inhibitor (NI) resistant virus by either mutations in the binding site of NA or mutations in the HA, which give rise to decreased binding affinity for cell receptors. As noted earlier, In the case of zanamavir and influenza A virus, NI resistance arises from mutations in position 119 with change of glutamic acid to glycine (E119G), or occasionally alanine or aspartic acid; or in position 292 with change of arginine to lysine (R292K). With oseltamivir, this same mutation can be selected for in vitro (Mendel and Sidwell, 1998). With influenza B and zanamavir, the glutamic acid to glycine in position 119 is selected in vitro, but similar resistant mutants have not been selected with oseltamivir. Mutations in the HA that may result in a reduced binding affinity of the HA to the cell receptors in MDCK cells are largely alpha-2–3 linked. However, in humans the respiratory tract cell receptors are alpha-2–6 linked.

Studies of post-treatment clinical isolates from oseltamivir and zanamivir trials in adults are showing that the emergence of virus resistant to these drugs is uncommon. Oseltamivir treatment studies provided sequential nasal swab samples from 716 patients randomized to receive treatment with placebo or oseltamivir at 75 mg BID or 150 mg BID for 5 days. The primary assay defined the NA enzyme inhibition phenotype of pre-treatment isolates and the last viral culture. A secondary assay looked for changes in viral genotype. No adequate phenotypic screen is available for HA, and therefore a genotypic assay was performed directly on the swab samples.

The sensitivity of viral NA to inhibition by the oseltamivir active metabolite was assayed for 621 patients (203 placebo, 419 treated, 87% of the culture positive patients in the studies). Four hundred fifty-two had matched pre and post treatment isolates. Only four patients, all of whom responded normally to treatment, harbored resistant virus on day 4 or 6 post-treatment. Thus, the incidence of NI resistance development was approximately 1% of patients. All four resistant viruses were of the H3N2 subtype; three mutations were R292K and one novel mutation (E119V) was identified. Supporting genotypic studies of NA ($n = 405$ sequences, 115 patients) confirmed that phenotyping had detected most resistant NA mutants. One additional case of a R292K mutation was found by genotypic assay in a group of 51 patients who shed virus to days 4–6 after treatment. The five patients who shed the resistant virus did not exhibit clinical deterioration. Clinical isolates with decreased sensitivity to NI were compared to the corresponding pre-treatment isolates from the same patient. The two mutant NA genotypes identified, R292K and E 119V, exhibited reduced infectivity and replicative ability in mice and ferrets. HA genotyping ($n = 80$ patients, 28 placebo and 52 treated) failed to identify treatment-related mutations even in those viruses carrying a mutant NA. Random HA variants were found in both the placebo and treatment groups at similar rates.

For zanamavir clinical trials, NA sensitivity studies have been performed on 59 post-treatment isolates and NA and HA sequencing on virus from 40 subjects (28 drug treated). There was no evidence for drug treatment related resis-

tance by any assay; however, few isolates beyond day 3 were assayed. A zanamivir resistant influenza Bisolate with mutations in NA and HA was obtained from an immunocompromised infant on zanamivir (Gubareva et al., 1998). A NA mutation in position 152 gave the virus a 1000-fold reduction in sensitivity to zanamavir, and a HA mutation (T198I) resulted in decreased binding to human cells. The mutant was less virulent than the corresponding wild type virus in ferrets.

In summary, resistance to NI can arise in both HA and NA. However it does not arise readily in vitro, and the clinical incidence appears to be low. Resistant viruses appear attenuated and, at least in current oseltamivir trials, clinical deterioration was not associated with the development of resistance. Overall, current studies suggest that neuraminidase inhibitors have an encouraging resistance profile.

6.3. Reassorting of influenza virus can alter both sensitivity to zanamavir and pathogenicity in vivo without the introduction of a mutation

Resistance of influenza viruses to neuraminidase inhibitors can develop as a result of mutations in the viral hemagglutinin that lower the affinity of the HA binding to the sialic acid receptor. This means that bound virus needs less NA activity for elution. A reassortant virus was obtained by co-infecting NWS^G /G70C and NWS^T /Tokyo influenza A viruses (the HAs differ by two amino acids). The NWS^T /G70C reassortant had no mutations, but was almost 100-fold less sensitive to zanamavir than either parent in a plaque reduction assay. It was also less sensitive to zanamavir in mice than the Tokyo parent virus, and had lower infectivity. Treatment of cells with purified NA showed that the G70C NA was more efficient at removing the receptors to which the NWS^T was bound. Resistance occurs because the NA can be inhibited by drug, but still retain sufficient activity to elute the virus. Thus, the balance between the HA binding activity and the NA eluting activity of each virus influences its sensitivity to zanamavir.

6.4. Short term prophylaxis with oral oseltamivir effectively prevents spread of influenza A and B

The family is an ideal environment for the transmission of influenza within the community and is, therefore, ideal for the study of antiviral drug efficacy. In order to test the efficacy of oral oseltamivir for preventing secondary spread of disease to close contacts of influenza infected cases, a randomized, double blind, placebo controlled study was carried out. In Europe, Canada and the US, 371 households with 955 contacts were identified prior to the influenza season with surveillance being used to identify the beginning of the outbreak. When an index case of respiratory illness (cough and coryza) was identified, oseltamivir (75 mg orally per day for 7 days) or placebo was given to family contacts, not to the index case. Forty-three percent of index cases were confirmed to have influenza infection and 99% percent of the contacts were treated within 48 h of onset of symptoms in the index case. There were no severe adverse events attributable to oseltamivir and minor adverse events were the same in the treatment and the placebo group.

During the week after exposure, oseltamivir reduced the incidence of clinical influenza caused by both influenza A (96% efficacy, $P = 0.001$) and B (78% efficacy, $P = 0.014$) as compared to placebo. There were 405 contacts (aged 12–85) of infected persons. In this group, the overall efficacy in reducing influenza A or B was 92% for contacts, and 89% for households ($P = < 0.0001$ vs. placebo). Viral shedding was reduced in these individuals by 82%. Oseltamivir was similarly efficacious in reducing the number of cases of influenza in contacts of households where the index case had a respiratory illness not caused by influenza, and where infection was most likely acquired outside the household.

Thus, post exposure treatment of household contacts with oseltamivir within 48 h of the onset of disease in the index case was well tolerated and prevented clinical influenza illness caused by both influenza A and B with an overall efficacy of greater than 90%.

6.5. Neuraminidase inhibitors in the elderly and other high risk populations

Influenza and pneumonia are leading causes of catastrophic disability and substantial health care costs in older adults. Vaccination is cost saving but hospitalization rates for pneumonia continue to rise. The clinical diagnosis of influenza in older adults is not reliable and the disability from influenza can be significant; cough, myalgias and malaise are commonly prolonged and the associated confinement to bed results in rapid loss of muscle power (as high as 2–3% per day).

In the nursing home environment, outbreaks of influenza are frequent with significant morbidity and mortality despite vaccination rates $\geq 80\%$ and a good vaccine match. Chemoprophylaxis until now has only been available for influenza A (amantadine and rimantadine), and major problems with viral resistance and drug toxicity in older people have limited their use for outbreak control. The neuraminidase inhibitors can overcome some of these disadvantages. An analysis of studies with zanamavir (10 mg BID) suggests that prior vaccination is required to realize benefit from zanamavir in the treatment of influenza illness in persons 65 years of age or older (Schilling et al., 1998).

Recommendations for preventing nursing home outbreaks should include staff and residents alike. Vaccination rates greater than 80% are desirable with use of antivirals for outbreak control for 14 days, or at least for 7 days after the last proven case of influenza (Drinka et al., 1998). Antivirals should not be offered to those who have been ill for more than 48 h, and if illness develops while receiving amantadine or rimantadine, it should be assumed that resistance has occurred; isolation of these cases is required.

Antiviral drugs are complementary and not an alternative to vaccination for preventing and treating influenza illness. Prophylaxis is more effective than treatment and should be optimized in the nursing home setting. Long term prophylaxis with antivirals might prove difficult because of the cost and side effects associated with drugs.

6.6. Oseltamivir is effective in the long term prophylaxis of influenza in vaccinated frail elderly

The safety and efficacy of oseltamivir (75 mg once daily) versus placebo over 6 weeks were studied in 548 nursing home residents in a multicenter, double blind, randomized, placebo controlled study. The mean age was approximately 81 years in both groups and about 80% were vaccinated against influenza prior to the influenza season. The drug was safe and well tolerated with side effects similar in both groups. Laboratory confirmed influenza with fever and respiratory or systemic symptoms occurred in 12/272 (4.4%) subjects in the placebo group, and in 1/276 (0.4%) in the oseltamivir group, for a protective efficacy of 92% ($P = 0.0015$). In those who had received vaccine earlier, laboratory confirmed influenza occurred in 11/218 (5%) placebo recipients, and in 1/222 (0.5%) subjects in the treated group (91% protective efficacy). Complications of influenza were diminished by oseltamivir in the intent to treat population; 2.6% of all subjects in the placebo group developed complications (bronchitis, pneumonia or sinusitis) versus 0.4% in the oseltamivir group (86% protective efficacy). Thus, oral oseltamivir effectively prevented clinical influenza illness in over 90% of treated vaccinated frail elderly individuals and the protection afforded was in addition to that provided by vaccination.

6.7. Efficacy of oseltamivir on H5N1 and H9N2 influenza viruses

The two potential pandemic viruses that have recently emerged and have been transmitted to humans are influenza A H5N1 and H9N2. H5N1 virus has not been recovered since 1997, but H9N2 (A/Hong Kong/1074/99) and its precursor (A/Quail/Hong Kong/G1/97) virus continues to circulate in birds in Asia. When new viruses enter human populations, vaccines are not available and antiviral drugs are critical for prophylaxis and therapy until vaccines can be prepared. GS4071 (the active metabolite of oseltamivir) inhibited the replication of H5N1 and H9N2 influenza viruses in MDCK cells with IC_{50} values ranging from 7.5 to 10 μM and the neuraminidase

enzyme activity of both viruses with IC_{50} values ranging from 7 to 15 nM. In murine models, H5N1 virus is lethal; but, for H9N2, larger doses of virus are required to cause death. Oral oseltamivir at doses of 1 and 10 mg/kg per day exerted protective efficacy against these viruses in infected mice; 1 mg/kg per day prevented death in 100% of mice infected with 5 LD50 of either H5N1 virus or H9N2 virus. When drug administration was delayed for up to 36 h after infection with H5N1 virus, oseltamivir was still effective.

Oseltamivir significantly reduced lung and brain virus titers in infected mice at doses as low as 0.1 mg/kg per day. Higher doses do not stop replication of H5N1 virus in the lung; however, doses of 100 mg/kg per day reduced lung viral replication of H9N2 virus to zero. The H5N1 virus that remained in lung tissue was not resistant. Oral oseltamivir in combination with rimantadine further reduced mortality from high challenge with H9N2 virus. Thus, oral oseltamivir reduces lung titers and prevents death in mice challenged with H5N1 or H9N2 virus and treatment up to 36 h after challenge prevents viral replication in the brain. The same studies were done with intranasal zanamavir and results were similar.

6.8. RWJ-270201: a novel neuraminidase inhibitor

RWJ-270201 is a novel neuraminidase inhibitor that was also developed using rational drug design. It has a novel cyclopentane ring structure which allows for extensive interactions with key residues in the neuraminidase active site. In vitro studies demonstrate that RWJ-270201 has potent and selective activity against influenza A and B neuraminidases (IC_{50} values < 11 nm) and against virus growth in tissue cultures. The in vitro pre-treatment of MDCK cells, followed by removal of drug from the supernatant, appears to have low inhibitory effect but effective activity was observed when treatment was begun ≤ 12 h post exposure. When compared to other neuraminidase inhibitors, RWJ-270201 demonstrates a lower IC_{50} against influenza (A H1N1) and influenza B strains than GS4071, and similar activity against (H3N2).

Oral dosing in murine models demonstrates efficacy for both prophylaxis and treatment of infection. A prophylactic regimen using 1 mg/kg per day begun 4 h prior to virus exposure and continued for 5 days post infection as BID dosing was efficacious in preventing influenza A and B infection in mice. Treatment regimens using 10 mg/kg BID for 5 days were efficacious in mice infected with influenza strains when the treatment was begun within 24 h after infection, and in mice infected with H1N1 virus, even when treatment was started up to 60 h after infection.

No cytotoxicity was observed with doses of 1000 μM in MDCK, embryonic African kidney or human lung carcinoma cells. No toxicity was seen in mice and rats with single oral doses up to 3000 mg/kg per day. Similarly, multiple doses of up to 1000 mg/kg per day for 5 or more days showed no effect.

Pharmacokinetic studies in humans demonstrate that RWJ-270201 is well tolerated by the oral route. It is rapidly absorbed, reaching sustained high plasma concentrations by 4–8 h. It has a long half life with a terminal phase plasma elimination of 12–25 h. The mean trough plasma concentration values exceed the median IC_{50} and IC_{90} for both influenza A and B. Elimination appears to be primarily renal. In Phase I clinical studies, subjects given RWJ-270201 exhibited no more adverse events than placebo recipients.

RWJ-270201 has recently been tested in an influenza A H1N1 human challenge model. The primary endpoint of the study was the reduction in area under the curve of mean viral titers over time. Preliminary results indicate that a significant antiviral effect was demonstrated with doses of 200 mg BID or 400 mg once daily. The mean duration of viral shedding was also reduced, particularly at higher doses.

Thus, RWJ-270201 is a novel neuraminidase inhibitor with a unique ring structure that shows potent antiviral activity against influenza A and B in vitro and in vivo models. It has been well tolerated in humans and a once daily oral regimen produced significant antiviral activity in a human challenge model.

6.9. Status of pleconaril and 3C protease inhibitors for rhinovirus

Rhinoviruses are the most common infectious pathogens of humans. They cause approximately 50% of acute upper respiratory tract illnesses and are associated with sinusitis, otitis media, and exacerbations of asthma and chronic lung disease. No antiviral agents of proven value are currently available. Intranasal interferon protects against infection but does not provide a therapeutic benefit.

Pleconaril is a drug that inserts into the hydrophobic pocket of the picornavirus capsid to inhibit attachment and/or viral uncoating. Pleconaril inhibits replication of most enteroviruses and over 93% of RV serotypes in vitro at concentrations obtainable with oral administration. Oral pleconaril appears therapeutically beneficial in adults and adolescents with viral meningitis and in human volunteers given experimental Coxsackie virus A21 infection (a respiratory virus) (Schiff and Sherwood, 2000).

A phase III clinical trial to determine the efficacy of pleconaril in the treatment of picornavirus respiratory illnesses was conducted in over 1000 adolescents and adults with at least one respiratory and one systemic symptom of less than 36 h duration (Hayden et al., 1999). Treatment was 400 mg BID or TID of pleconaril or placebo for 7 days. No difference occurred between placebo and treatment groups in adverse events.

The primary endpoint of efficacy was the time to complete resolution of five key respiratory and systemic symptoms. The BID pleconaril group did not differ from the placebo group, but TID pleconaril reduced the median duration of symptoms and recovery from illness by 3.5 days (25% reduction). The secondary endpoints of total severity of symptoms and specific individual symptoms were also reduced in the TID treatment group compared to placebo. Treatment benefit was detected within 1–2 days of treatment. These results suggest that pleconaril can reduce the symptoms of respiratory illness due to picornavirus infection.

Rhinovirus 3C protease is an appealing target for antiviral activity because it has an essential role in viral replication and has little similarity to mammalian enzymes. The crystal structure of 3C protease was determined in 1994. Because the active enzyme site is highly conserved, 3C protease inhibitors are likely to be active against all rhinovirus serotypes. The inhibitor being used in clinical trials is AG7088, a peptidomimetic inhibitor with a mean EC_{90} of 82 nm against a range of rhinovirus serotypes.

Intranasal AG7088 has been studied in experimental rhinovirus infection of susceptible adults for prophylaxis. AG7088 or placebo was administered 6 h before virus exposure and continued 2 or 5 times a day for 5 days. For early treatment, administration was begun 24 h after the challenge and continued for 5 days. Most adverse events were mild in severity and only a few, such as nausea and taste disturbance, were considered related to drug.

The proportion of individuals shedding virus was reduced in the two groups given AG7088 prophylaxis, and the mean virus titer of the area under the curve (AUC) of shedding was significantly reduced in the five times a day group. The incidence of colds, total symptom scores, respiratory symptoms and nasal discharge weights were also reduced, with a trend for greater effects in those receiving drug five times a day. In the early treatment study, AG7088 reduced nasal viral titers by day 2 or 3, and the reduction in AUC in the AG7088 group was highly significant compared to placebo. Total symptom scores, respiratory symptoms, and nasal mucus weights were significantly reduced when compared to placebo (23, 22 and 40%, respectively).

Intranasal AG7088 was safe and associated with a significant antiviral effect. It did not prevent experimental RV infection but it did moderate illness severity when initiated either before or within one day of infection.

6.10. Role of corticosteroids in treating respiratory virus disease

Glucocorticosteroids have a wide range of anti-inflammatory and immunosuppressive effects in many organ systems. They up regulate adhesion molecules such as ICAM-1, inhibit traffic of leukocytes to sites of inflammation, block release

of IL-1, IL-6, and TNF alpha from macrophages, and block the production of IL-2, IL-3, IL-6 and interferon gamma. In addition, they depress the release of prostaglandins and leukotrienes and affect pro-inflammatory arachidonic metabolites. During recent years there has been an increased understanding of the mechanisms whereby steroids inhibit the inflammatory reaction. By inhibiting nuclear factor kappa B, steroids inhibit a number of inflammatory reactions induced by viruses.

In 1992, the Infectious Diseases Society of America recommended steroids for viral respiratory illness only for croup (laryngotracheobronchitis). Croup is caused by PIV 1, 2 or 3 in 70% of cases. A metaanalysis of 24 studies of corticosteroids in the treatment of croup, concluded that corticosteroids are effective in improving the outcome of croup when given within 6 h of the onset of symptoms (Ausejino et al., 1999).

Bronchiolitis is the prototype disease of RSV infection and corticosteroids are not recommended. In the 1970's the AAP stated that there was no scientific basis for their use in bronchiolitis. Only one of several studies in the 1990s showed benefit in reduction of daily symptom severity score with steroid treatment. Most centers at this time do not use steroids routinely for RSV bronchiolitis. However, steroids are the mainstay of therapy in children with bronchial asthma, an entity induced in most cases by viral infections.

With the hypothesis that blocking the inflammatory cascade with the use of intranasal steroids could affect the symptoms of the common cold, a prospective, randomized, placebo controlled study was designed to administer intranasal fluticasone propionate treatment or placebo in 200 otherwise healthy young adults for 6 days beginning 24–48 h after onset (Puhakka et al., 1998a). Approximately 75% of cases were positive for a viral etiology, with rhinovirus being the isolate in 53% of cases. No recognizable clinical effects were observed in the severity of common cold symptoms in patients treated with fluticasone propionate when compared to placebo. Viral sinusitis was present in 57% of patients during the study period, but all resolved spontaneously. In the RV positive cases, there was a tendency for reduced incidence of sinusitis on day 7 of illness in the fluticasone group when compared to placebo (18 vs. 35%, respectively) (Puhakka et al., 1998b). 36% of fluticasone propionate recipients had positive RV cultures on day 7 of the illness, compared to 14% in the placebo group. The statistical significance of this finding disappeared when PCR results were included in the analysis. A study of intranasal fluticasone therapy of colds in children also found higher frequencies of rhinovirus shedding and otitis media when compared to placebo.

In summary, steroids are effective in the treatment of croup. Most agree that they are not effective in the treatment of bronchiolitis or the common cold. They appear to increase rhinovirus shedding. Future studies should consider the use and efficacy of combination therapy of antivirals with corticosteroids.

7. Summary

Viruses are the leading cause of respiratory infections in children and adults and are a major cause of morbidity and mortality worldwide. A variety of clinical syndromes and illness severity's result from viral respiratory infections reflecting the biologic differences of the various viruses as well as differences in host resistance. Infection with one of the viruses is the principal cause of serious diseases such as sinusitis, otitis media, bronchiolitis, pneumonia and exacerbations of chronic pulmonary conditions such as asthma. Young children, older adults, and those with underlying chronic disease are at particular risk for significant morbidity with infection. Patients with underlying immunodeficiencies, such as those infected with HIV and recipients of organ transplants, may also experience serious illness. Moreover, these persons have a reduced ability to respond adequately to vaccine.

The epidemiology of influenza virus is constantly undergoing change. New influenza A (H3N2) strains with the potential to infect humans were discovered in 1998 to be widespread in swine in the US. Also, for the first time, an influenza B virus was detected in harbor seals in Europe. The human outbreak of influenza A

(H5N1) virus that arose from infected birds in Hong Kong in 1997 was a clear example of a potential pandemic threat. In 1999, another new influenza A virus (H9N2) emerged in China where it caused disease in chickens; and two children in Hong Kong were discovered to be infected and ill with this virus. In addition to underscoring the need for improving and enhancing global viral surveillance, recent events have indicated a need for better training of personnel, availability of adequate laboratory facilities, and development of pandemic preparedness plans in different regions of the world. In this regard, the WHO has the roles of maintaining a global influenza surveillance network during interpandemic periods and of aiding countries in pandemic preparedness. Providing effective vaccination remains the principal intervention in a pandemic plan. However, the availability of newer antiviral agents effective against both influenza A and B (in addition to the currently available antivirals), offers the possibility of treatment of selected cases and use of short-term prophylaxis during a pandemic, particularly in regions of the world where time for development and use of vaccines will not be feasible.

The possibility of treating influenza has increased the demand for virologic diagnosis. Although viral culture remains essential for diagnostic and epidemiologic purposes, rapid diagnostic tests based on antigen detection that are specific and relatively sensitive for identifying both influenza A and B viruses are now available for use in the clinical setting. Genome amplification, by PCR and RT-PCR, has the greatest sensitivity but is more technically demanding than the widely available immunofluorescence and ELISA assays. A new method of diagnosis currently showing promise is TaqMan® PCR, a real time, quantitative PCR technique that offers rapid results, good sensitivity, and is less prone to contamination. Preliminary studies have shown promising results for the determination of viral loads in cystic fibrosis patients.

Genome amplification methods are also useful for the study of the epidemiology of respiratory viruses. Fragments of RNA recovered form victims of the 1918 influenza pandemic with the use of RT-PCR have shown the presence of avian-like HA and NA sequences but a clear mammalian origin phylogenetically, suggesting that the 1918 influenza virus was an avian H1N1 virus that underwent mammalian adaptation. Although reported by others, pantropism and neurotropism were not confirmed by RT PCR assays of other organs at the Armed Forces Institute of Pathology in the USA.

Respiratory viruses play a significant role in the pathogenesis, clinical course, and outcome of upper respiratory tract illnesses such as sinusitis and otitis media. Respiratory syncytial virus, rhinovirus, parainfluenza viruses 1, 2, and 3, and adenovirus are important causes of these illnesses in children and adults during the winter months. Adenoviruses are also notable as an important cause of disease that can affect many different organ systems. Viral replication in the respiratory tract results in the stimulation of multiple pathways for inflammation including cytokines and inflammatory mediators that lead to mucocilliary damage, dysfunction, and clinical symptoms. The use of combination anti-inflammatory and antiviral (interferon) therapy was of benefit in treatment of rhinovirus common colds. No benefit has been demonstrated with the use of steroids in viral respiratory illnesses, other than for croup in children. Pleconaril, a compound inhibiting receptor binding of picornaviruses, was beneficial in the treatment of acute rhinovirus infections in adults and adolescents and in experimental respiratory Coxsackie virus A21 infection in volunteers. AG7088, a 3C protease inhibitor, was shown to reduce infections or severity of illness when administered before or early in the course of infection.

The most significant breakthrough in antiviral treatment this past year was approval of the neuraminidase inhibitors (NI) zanamavir and oseltamivir. Both agents were approved in 1999 in the USA and many European and South American countries for the treatment of influenza A and B infections. They reduce the severity and duration of symptoms of influenza when administered within the first 2 days after illness onset. They are safe and generally well tolerated and the development of resistance is infrequent. Resistant viruses

occur late in about 1% of infected subjects by either a mutation in the binding site of NA or a mutation in the HA that reduces binding affinity and the need for NA activity. Alternatively, resistance may be seen where the balance of HA binding affinity and NA eluting activity of viruses without mutations is such that sufficient NA activity remains in the presence of drug. So far, no clinical deterioration has been associated with the development of resistance, and resistant viruses appear to be less virulent in animal models.

The two potential pandemic viruses that have recently emerged, influenza A H5N1 and H9N2, are inhibited in vitro and in animals by the NI drugs. In clinical studies, oseltamivir was shown to prevent the spread of influenza A and B to household contacts when administered after exposure to an ill family member. It also effectively prevented clinical influenza in vaccinated frail elderly populations when administered as long-term prophylaxis in the nursing home setting and, in doing so, provided additional protection to that provided by vaccination alone. Approval of these agents for prophylactic use against influenza A and B infections should occur soon. Newer but similar compounds are also under development; RWJ-270201 is a novel NI with a unique cyclopentane ring structure that shows potent activity against influenza A and B in vitro and in animal models. It has been well tolerated and shown to have an antiviral effect in human challenge studies.

The most important intervention for the control of viral infections and their complications is prevention through immunization. Significant advances have occurred recently in the development and use of antiviral vaccines. The live attenuated cold-adapted influenza vaccine is now updated annually to match the FDA recommendations for the trivalent inactivated vaccine and is produced consistently to a viral titer that, when administered intranasally to children or adults, has resulted in immunity to the vaccine strain and to drift variants. An ongoing study seeks to determine whether universal immunization of young children with the cold-adapted vaccine will significantly reduce influenza in a community.

Methods to improve on the currently available inactivated influenza vaccine in high risk groups such as the elderly, and for use before exposure to a pandemic virus are under investigation. The immunogenicity of the currently available trivalent inactivated vaccine was enhanced by supplementation with recombinant NA (rNA) in animal models and in early studies of human experimental infection. The supplemented vaccine was safe, immunogenic, and followed by decreased symptomatology and viral shedding. An MF-59 adjuvanted influenza A (H5N3) vaccine was more immunogenic in naive volunteers than standard aqueous vaccine. Vaccines to augment CTL memory T cells to enhance protection against pandemic and interpandemic influenza virus infection, and production of attenuated vaccine strains via reverse genetics to modulate interferon sensitivity are other new vaccine options. Application of reverse genetics to production of vaccines for RSV and PIV is permitting genotypic and phenotypic manipulations with relative ease. Early results have provided promising new candidate vaccines.

Preliminary results with cold adapted-temperature sensitive RSV and PIV live attenuated vaccines in young children indicate these vaccines are safe and immunogenic in this population. As an alternative, a novel recombinant RSV subunit vaccine, BBG2Na, was shown to be immunogenic and protective in mice, and to be safe and immunogenic in RSV seropositive healthy adults. Parallel studies to define the immune correlates of RSV disease and the factors contributing to the severity of disease in younger infants are ongoing. The identification of T-cell epitopes in RSV and clarification of their role in immunopathogenesis and as vaccine targets is an important effort.

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