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Review

Current research on respiratory viral infections: Fourth International Symposium

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1. Introduction

The Fourth International Symposium on Respiratory Viral Infections was convened by The Macrae Group (New York, NY) in Curaçao, Netherlands Antilles on 29 November–2 December, 2001. This symposium provides an annual forum for virologists, vaccinologists, clinicians, pharmacologists, and public health specialists to discuss recent advances in respiratory virus research in an interdisciplinary fashion (Kaiser et al., 1999; Munoz et al., 2000; Schmidt et al., 2001). The spectrum of discussion ranged from basic virology and pathogenesis to epidemiology, immunology, and management strategies, with particular attention to vaccines, antivirals, and economic issues.

The meeting was chaired by Frederick Hayden (University of Virginia) and Albert Osterhaus (Erasmus University). Additional members of the meeting's Scientific Advisory Board were Robert Belshe (St. Louis University), Robert Chanock (National Institutes of Allergy and Infectious Diseases), Robert Couch (Baylor College of Medicine), John Mills (Burnet Institute), Peter Openshaw (St. Mary's Hospital), and Maria Zambon (Public Health Laboratory Service). Invited speakers included: Larry Anderson (Centers for Disease Control and Prevention), Reinhard Glück (Berna), Jack Gwaltney, Jr. (University of Virginia), Frederick Hayden (University of Virginia), Terho Heikkinen (University of Turku), Sebastian Johnston (National Heart and Lung Institute, Imperial College of Science, Technology, and Medicine), Arnold Monto (University of Michigan), Kristin Nichol (Minneapolis Veterans' Administration Medical Center), Peter Openshaw (Imperial College School of Medicine at St. Mary's), Albert Osterhaus (Erasmus University), Peter Palese (Mt. Sinai School of Medicine), Amy Patick (Agouron Pharmaceuticals, Inc.), Dan Pe-

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vear (ViroPharma, Inc.), Ann Reid (Armed Forces Institute of Pathology), Guus Rimmelzwaan (Erasmus University), Ken Shortridge (University of Hong Kong), Thomas Szucs (Hirslanden Holdings), Theodore Tsai (Wyeth Pharmaceuticals), Ed Walsh (Rochester General Hospital), Birgit Winther (University of Virginia), Maria Zambon (Public Health Laboratory Service). The newly sponsored Roche Young Investigator Awards for 2001 went to Guy Boivin (Laval University, Québec), Jacco Boon (Erasmus University, Rotterdam), Boris Ferko (Institut für Angewandte Mikrobiologie, Vienna), and Jonathan McCullers (St. Jude Children's Research Hospital, Memphis). In addition, there were 15 oral and 31 poster late breaker presentations, as well as two question and answer sessions and a poster review session conducted by Albert Osterhaus.

Educational grants for this meeting were provided by Agouron Pharmaceuticals, Inc., a. Pfizer Co., Biota Holdings Ltd., Eli Lilly and Co., GlaxoSmithKline, ID Biomedical, Janssen Cilag, MedImmune, Inc., Ortho-McNeil Pharmaceutical, The R. W. Johnson Pharmaceutical Research Institute, Solvay Pharmaceuticals B.V., ViroPharma, Inc., and Wyeth Lederle Vaccines.

The keynote address to start the meeting was delivered by Dr Jack M. Gwaltney, Jr (University of Virginia) and entitled 'Life with Rhinoviruses.'

2. Epidemiology and impact

2.1. Life with rhinoviruses (Jack Gwaltney, University of Virginia, Charlottesville, VA)

Over what is now a 40-year career, I have enjoyed a sustained interest in acute respiratory disease and specifically in rhinovirus. I graduated from the University of Virginia School of Medicine in 1956. That same year, Dr William Mogabgab, working at the Great Lakes Naval Training Station, reported the discovery of the first rhinovirus strain, 2060, later numbered as rhinovirus type 1A. (Pelon et al., 1957) Earlier, there had been important epidemiologic studies into colds conducted in England by Drs Christopher Andrewes and David Tyrrell and in the US, but these studies were seriously hampered by a lack of virology (Frost and Gover, 1932; Andrewes, 1965; Dingle et al., 1964). The discovery of rhinovirus added greatly to the ability of investigators to study colds and also became an important influence on my career. Following a residency in Internal Medicine at Case Western Reserve University, I entered the US Army Medical Corps in 1960 and was stationed at Fort Dix, NJ where I was responsible for the care of many hundreds of recruits with adenovirus ARD. There I observed the research that was being conducted on ARD by Drs Harry Rose of Columbia University and Edward Busher of Walter Reed Army Institute of Research using the new and exciting technique of tissue culture. In 1962, I returned to the University of Virginia to join Dr William Jordan, an author of the highly regarded Cleveland Family Study of Minor Illnesses in the Home. Dr. Jordan had moved to Charlottesville to Chair the Department of Preventive Medicine and was continuing to conduct research on the common cold. During this period, Dr Leonard Hayflick of the Wistar Institute developed the WI 26 strain of human embryonic lung cells which were found to grow rhinovirus very efficiently and were ideal for epidemiologic studies (Hayflick and Moorehead, 1961).

Dr Jordan and I began a study of acute respiratory disease in the approximately 500 employees of the Eastern Regional Office of the State Farm Insurance Company. This work was supported by the Commission on Acute Respiratory Disease of the Armed Forces Epidemiology Board and the Vaccine Development Branch of the National Institutes of Allergy and Infectious Diseases. The insurance employees maintained daily symptom records and supplied specimens for viral culture and serology. The study eventually continued for 15 years. A nurse monitored the employees on a weekly schedule to ensure accurate recording of data. In this study, which employed cell culture for viral identification, rhinovirus accounted for 25% of colds, giving a rate of 0.77 rhinovirus colds per person per year (Gwaltney et al., 1966). This attack rate was consistent with that of an earlier study of young adults conducted in

Chicago by Dr Dorothy Hamre (Hamre et al., 1966). The age-specific rates of infection were higher in children than in adults. A seasonal variation in rhinovirus attack rates was also observed in the Charlottesville population which resulted in a consistent large fall peak of illness associated with rhinovirus. Of interest, this yearly rhinovirus outbreak coincided in time with a recurrent peak of respiratory illness identified 20 years earlier by Dr Wade Hampton Frost, a graduate of the University of Virginia and the first Dean of the School of Public Health at Johns Hopkins University. Details of the daily symptom patterns of rhinovirus colds were also obtained from the insurance company data (Gwaltney et al., 1967).

Serotyping of the viral isolates became a great challenge as more and more different rhinovirus types were discovered. Because early plastic microtiter plates were toxic to cells, a system was developed that used the bottoms of small glass tubes to provide wells for growing the cell cultures (Gwaltney, 1966). Rhinovirus serotyping was eventually done using 10 serum pools put together in a combinatorial system and new serotypes were identified in Charlottesville (Gwaltney et al., 1968). Epidemiologic studies in the US and England lead to the discovery of many new rhinovirus serotypes, the number eventually reaching 100 (Kapikian et al., 1967, 1971; Hamparian et al., 1987).

In 1970, I recruited Dr J. Owen Hendley who joined me in the work. With the availability of viral serotyping, an earlier attempt had been made to trace the spread of individual virus types through the insurance company population. It became clear that the different strains did not typically spread among people who were working together in the same area of the office. This suggested that the employees were becoming infected at home. As a result, we turned our attention to rhinovirus spread among families of employees in their homes (Hendley et al., 1969). This work led to the finding that children were usually the first to develop rhinovirus colds in the family as a result of exposure at school. The sick child then transmitted the infection to other family members. Also, serologic findings from this study when combined with data from a study of experimentally infected volunteers revealed the levels of neutralizing antibody that were necessary to protect against infection following natural exposure (Hendley et al., 1972).

Now that it was clear that certain, as yet undetermined, conditions in the home promoted spread of rhinovirus in the family, we turned our attention to trying to understand what these conditions were. The first evidence bearing on rhinovirus transmission came about through serendipity. While conducting an earlier study on the appearance of the nasal mucosa in infected and uninfected subjects using a dissecting microscope to view the nose, I observed that infection had accidentally spread to control subjects by way of contaminated nasal specula. This focused attention on the possible mechanisms for accidental introduction of virus directly into the nose by the types of intimate activities that take place among family members. It was discovered that cold patients with rhinovirus in their nasal secretions also frequently had virus on their fingers (Hendley et al., 1973). When these same patients sneezed or coughed onto petri dishes containing viral collecting broth which was then cultured, virus was infrequently recovered. This directed attention away from viral spread by large and small particle aerosol and to the importance of contaminated hands. We then conducted experiments in which subjects' hands were intentionally contaminated with rhinovirus, following which it was observed that virus could be efficiently transferred to the hands of other subjects by brief hand-to-hand contact. Rhinovirus on the skin of the fingers was shown to be readily transferred to the mucous membranes of the nose or the eye by means of finger contact with these sites. Rhinovirus was also found to survive well on human skin and environmental surfaces. Finally, the number of finger-to-nose and finger-to-eye contacts which occurred naturally in routine activities such as medical grand rounds and Sunday school class were recorded.

A more elaborate set of experiments was then planned to further explore the feasibility of the hand-contact/self-inoculation route of rhinovirus transmission (Gwaltney et al., 1978). In this work,

subjects (donors) were experimentally infected with rhinovirus and then exposed to other susceptible volunteers (recipients) by one of three types of exposure. In the first type of exposure, the donors and recipients had brief hand-to-hand contact following which the donors touched their nasal and conjunctival mucosa. In the second type of exposure, recipients were exposed to donors across a small table while the donors coughed, talked loudly and sang in an effort to create a large particle aerosol. In the third type of exposure, the donors and recipients were housed continuously for 3 days in the same room, separated by a double wire partition that allowed transmission of small particle aerosols throughout the room. Eleven of 15, 1 of 12, and none of 10 recipients in the respective types of exposure became infected. These findings demonstrated that under the controlled conditions of the experiment, small and large particle aerosols were considerably less efficient in the spread of rhinovirus than exposure by the hand-contact/self-inoculation route.

With hand contact implicated as a possible route of rhinovirus transmission, prevention became the next issue of interest. Studies were conducted in which rhinovirus was applied to subjects' hands and the hands were then treated with a variety of different types of commonly used sterilizing agents (Hendley et al., 1978). Washing the hands with soap and water was found to effectively remove rhinovirus. However, most of the chemical agents tested did not provide the desired long-lasting virucidal activity on the skin. Solutions with low concentrations of elemental iodine were found to be the most effective and were able to keep hands sterile for up to 4 h (Carter et al., 1980). Using iodine hand treatment as a method of prevention, a controlled blinded contact prophylaxis study was then done in which mothers of families were instructed to use iodine to treat their hands on a regular basis when a family member was recognized as having a new cold (Hendley and Gwaltney, 1988). A control group of mothers treated their hands with a solution containing brown food dye with a small non-virucidal amount of iodine. Approximately 50 families were studied each year from 1979 through 1982. Four of 58 (7%) of mothers in the iodine hand treatment group compared to 16 of 79 (20%) of those in the placebo group developed colds after exposure to an index case of the common cold. None of 11 of the iodine treated patients compared to 5 of 16 (31%) of placebo patients became infected with the rhinovirus type that was introduced into the home by the index case. By interrupting viral transmission by treating the hands, it was demonstrated that the hand contact/self-inoculation route of rhinovirus transmission was not only feasible but occurred under natural conditions (Gwaltney et al., 1978). Thus, it appeared that accidental rhinovirus introduction into the nose could be prevented by hand washing and avoidance of finger-to-nose and finger-to-eye contact and was probably of practical value in avoiding colds.

In the 1980s, we also began studies on the pathogenesis of rhinovirus colds (Turner and Gwaltney, 1984). It was well known that rhinovirus resulted in an obvious cytopathic effect in cells in cell culture, and it was assumed that similar changes occurred in the human nasal mucosa of persons with colds, although pathogenic events in humans had received little attention. In 1970, Dr Gordon Douglas had made the important discovery that the amount of rhinovirus required to infect a human was very small. One human infectious dose 50 of rhinovirus could be as little as one tissue culture infectious dose 50 (Douglas and Couch, 1969). Another important finding to emerge from the rhinovirus challenge experiments in Charlottesville and elsewhere was that in susceptible (antibody-free) people, the nose has virtually no protection against the virus once it was deposited on the nasal mucosa. For example, in Charlottesville, an infection occurred in 95% of 243 antibody-free subjects who were challenged intranasally with rhinovirus (Gwaltney and Hayden, 1992). Of interest, only 74% of the infected subjects developed symptoms of a cold. A similar rate of inapparent infection had been observed with natural rhinovirus infection (Gwaltney, 1997).

As work on rhinovirus pathogenesis progressed, it was determined that the initial step of virus introduction into the nose or the eye (from where

it goes down the lacrimal duct into the nose) was followed by transport of the virus to the posterior pharynx (adenoid) by the ciliary epithelium of the nasal mucus membrane (Winther et al., 1986). Dr Birgit Winther, who had joined the Charlottesville group, and Dr Niels Mygind of Copenhagen were lead investigators in this work. Infection then appeared to be initiated in the posterior pharynx in the lymphoepithelial cells which are present in the crypts of adenoidal tissue. These cells were shown to be rich in ICAM-1, the major cellular receptor for rhinovirus (Winther et al., 1997). In another experiment designed to investigate the time sequence of rhinovirus infection, production of new virus in the upper airway was shown to occur rapidly after viral inoculation into the nose with new virus being recognized within 8–10 h after intranasal inoculation (Harris and Gwaltney, 1996). Symptoms were also observed to begin early, within 10–12 h after viral inoculation. These findings indicated that to achieve optimum benefit from treatment for rhinovirus colds, dosing should be started as early as possible after the onset of symptoms is recognized by the affected person. Recent work by Dr Eurico Arruda and associates has shown that most persons can accurately determine they have contracted a cold within 16 h after the first symptoms appear (Arruda et al., 1997).

Another important finding to emerge from the pathogenesis studies in Charlottesville was that, unlike in cell culture, rhinovirus did not cause a significant amount of cytopathology in the cells of the nasal mucosa (Winther et al., 1986). Examination of nasal biopsies showed that it was difficult, if not impossible, to detect histologic change in nasal mucosa taken from subjects with rhinovirus colds. This finding suggested that the pathogenesis of rhinovirus colds was, in large part, associated with the activation of various inflammatory events in the immune system of infected persons. Over the past decade, the role of inflammatory mediators in the pathogenesis of rhinovirus colds has received increasing attention. The investigative approach to this problem has included the measurement of mediators in the nasal fluid of persons with colds, observations on the occurrence of the different cold symptoms in volunteers after intranasal challenge with known inflammatory compounds, and attempted blockade of symptoms in infected subjects by the use of anti-inflammatory compounds with known mechanisms of action. This work has led to the discovery of multiple inflammatory events which may have a role in rhinovirus pathogenesis (Gwaltney and Rueckert, 1997). These include the inflammatory mechanisms associated with the release of histamine, kinins, prostaglandins and interleukins, and the activity of the parasympathetic nervous system.

The traditional treatments for colds which have been directed at symptom management have obviously worked by blocking some of the inflammatory pathways that have been recognized. The alpha adrenergic activity of oral and topical decongestants shrinks the mucosal swelling of the nasal turbinate which is the cause of nasal obstruction. First generation antihistamines work through their H1 blocking and anticholinergic activity to reduce the seromucous gland secretion that contributes to rhinorrhea and to suppress the sneeze reflex, (Gwaltney et al., 1996; Turner et al., 1997; Gwaltney and Druce, 1997) the latter effect possibly occurs at a central location (Muether and Gwaltney, 2001). Non-steroidal anti-inflammatory drugs reduce sore throat, suppress cough, (Sperber et al., 1992; Insel, 1996; Nozhat et al., 1990; Fogari et al., 1992) and reduce systemic complaints such as malaise. The suppressive effect on cough may be due to anti-prostaglandin activity since some prostaglandins are potent stimulators of cough (Doyle et al., 1990).

The discovery of compounds with antiviral activity against rhinovirus has provided a more basic approach to treating colds. In 1978, I had been fortunate to recruit Dr Frederick Hayden to join the Charlottesville group. Dr Hayden had a particular interest in antiviral compounds to treat respiratory infections. We and others have tested several of these compounds in humans, going back to an unpublished clinical trial I conducted for SmithKline and French Laboratories with a triazinoindole compound in 1972 (Gwaltney, 1970; Hayden and Gwaltney, 1984, 1982; Turner et al., 1999). However, the results of the clinical trials have been disappointing because of the

modest therapeutic effects that the antivirals have provided. For that reason and because of the recognized role of inflammatory events in the pathogenesis of rhinovirus colds, I turned my attention to cold treatments which combine an antiviral compound with one or more anti-inflammatory compounds. This approach showed promise in a proof of concept study and is currently my major interest (Gwaltney, 1992). A trial was recently completed in the rhinovirus challenge model using a combination treatment composed of intranasal interferons plus oral chlorpheniramine and ibuprofen (Gwaltney et al., submitted for publication). This combination was effective in lowering the severity of total and individual symptom scores and in reducing the amount of nasal mucus production and nasal tissue use. The short course of intranasal interferon used in the treatment avoided the nasal irritation which occurs with prolonged interferon administration.

Work in another area of rhinovirus pathogenesis, demonstrated in 1994 by sinus CT examination that abnormalities in the paranasal sinuses, which are the result of collections of thick adhesive exudates, occur in a high proportion of patients with colds (Gwaltney et al., 1994). This shows that colds are a viral rhinosinusitis not just a viral rhinitis. In a recent follow-up study to these findings, it was shown that nose blowing propels nasal fluid from the middle meatus into the sinuses and probably accounts, at least in part, for the sinus abnormalities observed in colds (Gwaltney et al., 2000b). How to prevent or remove the viscous, adhesive material that collects in the sinus during colds is an area of current interest.

I look back at a career that has spanned the discovery of rhinovirus in 1956, the elucidation of its epidemiology in the 1960s and 1970s, the beginning attempts to understand its pathogenesis in the 1980s, the solution of its atomic structure by Rossmann and associates in 1986, (Rossmann et al., 1985) and now therapeutic approaches with antivirals and combined cold treatments. It has been an exciting story to follow and a privilege to have worked in a field from its very inception to the time when it has reached maturity. This by no means implies that the story is over. Work in the field is being continued by a small but talented and dedicated group of investigators, many of whom are also personal friends. That is the advantage of a small family. These investigators are actively adding to the findings that have been reviewed here. I believe that before long, an answer can be given to that tiresome old question, 'Why can't you cure the common cold?' And the answer will be, 'we did.'

2.2. Changing epidemiology of RSV (Larry Anderson, Centers for Disease Control and Prevention, Atlanta, GA)

Respiratory syncytial virus (RSV) has long been considered an important cause of serious lower respiratory tract disease in infants and young children throughout the world and as such an important target for vaccine development. Over the last decade, it has also become clear that RSV is also an important pathogen in other age groups and our thinking about treatment and prevention needs to include the infant and young child, those with compromised cardiac, immune, and respiratory systems of any age, and the elderly. Unfortunately efforts to develop RSV vaccines have not yet been successful, and sporadic prevention of RSV disease is limited currently to RSV immune globulin or a humanized murine monoclonal antibody as prophylaxis for serious RSV disease in the premature infant and the infant with chronic lung disease. In the US, RSV causes yearly outbreaks that usually begin in late fall or winter and last until late winter or spring. These outbreaks occur as community and not regional or national outbreaks as illustrated by variation in timing of outbreaks in communities and differences in outbreaks strains between communities. RSV outbreaks are consistently associated with substantial increases in hospitalizations for bronchiolitis and pneumonia in children < 1 year of age. Using national hospital discharge diagnosis data and death certificate data, we estimate that RSV is associated with 73 000–126 000 bronchiolitis and pneumonia hospitalizations and 171–510 deaths in children < 1 year of age each year. Community RSV outbreaks also bring a risk for nosocomial outbreaks that can be of substan-

tial concern for hospitalized persons of all ages but especially in patients with compromised immune systems. Recipients of bone marrow transplant have been reported to have mortality rates over 50% with RSV pneumonia. Molecular epidemiologic studies have demonstrated that RSV nosocomial outbreaks often result from multiple introductions of distinct strains from the community.

Over the last decade the importance of RSV as a cause of serious lower respiratory infection (LRI) in elderly patients has become increasingly appreciated. A study of community acquired LRI in those over 18 years of age in Ohio, suggested that RSV is associated with 3–5% of LRI hospitalizations during the RSV outbreak season in all ages. Applying rates of RSV detections from prospective studies of LRI in adults to national hospital discharge diagnosis and death certificate data, we estimate that RSV leads to 14 000–62 000 hospitalizations and 1500–6700 deaths each year in the US in persons $\geqslant 65$ years of age. In unpublished data, we obtained similar results for RSV-associated deaths in the US from a model that uses virus detection surveillance data and multiple cause of death data to estimate RSV and influenza-associated deaths. This model suggests that RSV may cause $\sim 50\%$ of the number of deaths that influenza does. A recent study using improved diagnostic methods, i.e. RT-PCR, supports the concept that RSV cause $\sim 50\%$ of amount of disease that influenza does in adult patients (Zambon et al., 2001b). In this study, RSV was detected at a rate that $\geqslant 50\%$ of the rate for influenza detection in patients with influenza-like illness.

One of the limitations, in defining the epidemiology of RSV disease in older children and adults is the ability to detect infection. Serology has been the most sensitive method to detect RSV infection in older children and adults. Virus isolation and antigen detection assays are likely to detect 50% of infections. Recently developed nested or other sensitive PCR-based assays, however, appear to give sensitivities closer to 75% compared to serologic studies and will make it possible to further characterize the epidemiology of RSV disease in elderly populations. Recently, we have applied PCR to studies of LRI in infants and young children and found that PCR may markedly improve RSV diagnosis in this population as well. In these studies, we nearly doubled the number of RSV positive specimens with a sensitive PCR assays as compared to tissue culture isolations studies.

2.3. Picornaviruses and acute otitis media (Tapani Hovi, National Public Health Institute, Helsinki, Finland)

The association of picornaviruses, rhinoviruses and enteroviruses, with acute otitis media (AOM) has been known for a long time. Recent studies have demonstrated that these viruses are associated with more than half of the cases in young children with AOM.

During the last several years, the National Public Health Institute (KTL), Helsinki, Finland, has participated in two large prospective studies on AOM in children younger than 2 years. In the first study, the FinOM Cohort Study, one of the primary questions was to see if different viral infections had different roles as risk factors for AOM. Whenever any of the 329 children had respiratory symptoms, samples for virus studies were collected. In the second study, the FinOM Vaccine Study, 2497 children received either a pneumococcal conjugate or hepatitis B vaccine, and the sample collection was limited to events when the study physician diagnosed AOM in the child. Samples that were collected included nasopharyngeal aspirate and middle ear fluid (MEF) at AOM, and in addition, paired sera in the Cohort Study.

In the Cohort Study, rhinoviruses were the most frequently encountered viruses (41%) in patients with AOM. After rhinoviruses, RSV, parainfluenza 3, influenza A, adenoviruses, influenza B, and parainfluenza 1 and 2 were found in association with AOM episodes. The frequency of influenza B and parainfluenza 1 and 2 was extremely low and enteroviruses were not screened (Vesa et al., 2001). Rhinoviruses were detected with a combined cell culture and RT-PCR method whereas antigen detection was used for the other viruses. Many infections were missed

with the latter technique, as a subgroup of AOM episodes studied with serology revealed significantly more cases (Räty et al., unpublished). In the second study, enteroviruses were also screened by direct RT-PCR and they were very common, second only to rhinoviruses. Together, rhinoviruses and enteroviruses were associated with more than 50% of all AOM episodes (Nokso-Koivisto et al., unpublished).

Further studies on rhinovirus serotypes were prompted by the epidemiological analysis of the FinOM Cohort study. This study found a strong correlation between HRV infections in the nasopharynx and HRV isolated from the MEF. There were two epidemics in which the virus was found relatively more frequently in the ears. This raised the question as to the possibility of specific ototropic strains. This question is difficult to answer because conventional serotyping of HRVs is extremely tedious and the study did not have a good collection of representative virus strains from different seasons.

An RT-PCR amplicon sequencing-based method for rhinovirus identification, similar to what has recently been established for enteroviruses, has been explored. This approach has targeted two genomic regions; a 549 nt motif coding for VP4 and part of VP2, and the VP1 coding region. The VP4/VP2 region was initially chosen as it was relatively easy to get working primers. Further work to develop primers for the VP1 coding region, which for enteroviruses was more reliable for serotype identification than the VP4/2 region, is ongoing.

Using phylogenetic analysis based on the VP4/VP2 sequences, all of the 102 HRV prototype strains clustered with the two known groups of human rhinoviruses, HRV A or B, except for one. This exception was HRV87 and appeared to be a closer relative to an enterovirus, EV70, than any of the other rhinoviruses. In general, the prototype strains were easily distinguishable from each other, although there were six pairs of serotypes relatively close to each other, including the currently unnumbered Hanks strain, which was very close to HRV21. Sixty-one field isolates collected during the FinOM Cohort Study were also included in the analysis. If we use the range of evolutionary difference between the prototype strains as a criterion for serotype, as many as 19 different serotypes might have been circulating among the about 300 children in a single suburb of Tampere from autumn 1994 to spring 1997. One of them may be a new serotype as there was no close-by prototype strain of the known serotypes (Savolainen et al., in press).

Detailed studies were conducted on the HRV87. The sequence of HRV87 in the VP4/VP2 region was even closer to the other HEV D member, EV68. EV68 appears to be a prime strain of HRV87 and is not neutralized by anti-HRV87 but capable of inducing cross-neutralization. These two viruses are definitely acid-sensitive, while EV70 is not and all strains appear to exploit DAF as a receptor in HeLa-Ohio cells (Blomquist et al., 1999). Both of these viruses were then sequenced, and two substrains of both of them, over three other regions. The VP4/VP2 region of the HRV87 and of the EV68 strains were very close and clearly the HEV D substrains clustered together with EV70. The HRV87 and EV68 also had similar VP1 and 3D regions, but the 5′ non-coding region of the two viruses was clearly distinct from EV70. EV70 is known to cluster together with HEV C and polioviruses in this region; HEV A and HEV B form another group and the two clusters of HRVs branches of their own.

From these studies, then, it is clear that all but one of the HRV prototype strains representing the 102 established serotypes clustered in the two known genetic groups HRV A and HRV B ($n = 76$ and 25, respectively). The exception was a HRV87 which appeared to share serotype identity with EV 68, a member of the HEV-D cluster. The HRV87/EV68 differ from EV70 by being acid labile like true rhinoviruses and by the unique 5′ NCR sequence, but share receptor specificity (DAF) in HeLa cells. The prospects of genetically identifying the HRV strains appear promising but will require technical improvements in VP1 sequencing. With further studies, new, unclassified HRV serotypes may be discovered.

3. Virology

3.1. Update on the 1918 influenza virus (Ann Reid, Armed Forces Institute of Pathology, Rockville, MD)

The infamous 1918 'Spanish' influenza had its origins in the spring of 1918, although the initial wave of infection was not very lethal in the US. During the late spring and early summer, the virus spread through Europe. A second wave started at the end of August on 3 continents and exploded into a worldwide pandemic in September through November 1918. The pandemic killed between 21 and 50 million people worldwide and over 675 000 in the US alone. The virus struck a particularly strong blow against the US troops and was responsible for 43% of the 100 000 American deaths during the entire war. The 1918 virus was powerful enough to cause a transient approximately 10-year reduction in average life expectancy in the US. The 1918 virus behaved very differently from other studied strains in that it caused a significant number of deaths in young adults, with a peak in age 25–34, in addition to causing increased morbidity and mortality among the very young and very old.

To better understand the pathogenesis behind the severity of infection, it was essential to analyze the virus, by recovery of viral RNA from autopsy specimens of patients who died during the pandemic secondary to acute viral pneumonia. Seventy-five samples were found but only 2 cases had evidence, by PCR, of virus RNA. The first patient was a 21-year-old private from Ft. Jackson, SC who died on September 26, 1918 after being sick for 6 days with influenza and pneumonia. The second patient was a 30-year-old private from Camp Upton, NY who died on September 26, 1918 after being sick for 3 days with influenza and pulmonary edema. A third case was eventually identified in an Inuit woman, exhumed from Teller Mission (now Brevig Mission), AK who died in a village in November 1918 in which over 85% of the adult inhabitants died within a week secondary to influenza. She had been ill for less than 5 days prior to dying. From lung samples, RNA was first extracted before being reverse transcribed into cDNA. Small fragments were amplified by PCR and then cloned and sequenced. These sequences were finally confirmed and compared to other known influenza strains. From these sequences, scientists hoped to understand where the virus came from, how it first infected man, why the virus was so lethal, and what genetic features were related to its virulent behavior or activation of the host response.

To date, the gene segments for the surface glycoproteins hemagglutinin and neuraminidase, and those of the internal proteins for non-structural, matrix, and nucleoproteins have been sequenced (Reid et al., 1999, 2000; Basler et al., 2001). Three polymerase genes are still being sequenced. The sequence analysis indicates that the hemagglutinin (HA) has no cleavage site mutations, as seen in the Hong Kong H5N1 or other virulent poultry influenza strains. The HA has a receptor binding pattern consistent with an avian origin and minimal mammalian adaptation. Most of the antigenic sites of the 1918 HA match the avian consensus. Phylogenetic analyses place the 1918 HA solidly within but very near the root of the mammalian clade, which makes it the most avian-like of all mammalian sequences. The phylogenetic analysis places the HA sequence near the intersection between human and swine influenza. By plotting the rate of amino acid change by year of the HA, it is clear that the 1918 strains lies very close to the predicted intersection between human and swine HA's. Analysis of the neuraminidase (NA) shows consistent results with those observed for HA. The HA and NA genes are almost certainly different from previously circulating strains and had not circulated widely in the human population before the pandemic.

Focus was then turned to the internal protein genes to better understand the virus. The 1918 matrix was sequenced and found to contain 1027 nucleotide segments with consensus M1 and M2 open reading frames. No mutations known to affect virulence were found, and the M2 transmembrane domain showed no sequence which would confer amantadine resistance. Phylogenetically, the matrix sequence was near the root of human and swine clades. The M2 extracellular domain has species-specific motifs of unknown

functional significance but which were used to better understand the source of this virus. The 1918 sequence matched subsequent human strains, consistent with having been retained from the previous circulating human strain. The 1918 sequence does differ one amino acid from other human sequences in the M2 extracellular domain.

Collaboration between researchers at St. Bartholomew's Royal London Hospital and the group at the Armed Forces Institute of Pathology has resulted in the identification of 5 additional cases from London in which influenza RNA from 1918 is present by RT-PCR in preserved tissue. The signal from three of these cases is very weak, so that detailed analysis was predominately conducted on the 2 stronger signals. The HA1 sequenced from these two cases is identical to the A/Brevig Mission/1/1918 virus, thus confirming the relative genetic uniformity of the pandemic virus.

Since the 1918 influenza virus appeared to be avian in origin, attention was turned to identifying specimens from wild waterfowl. With the help of Dick Slemons and James Dean, 25 wild waterfowl, representing 16 species, that were collected between 1915 and 1920 and which were catalogued and preserved in ethanol were identified at the Smithsonian Institute (Washington, DC). Cloacal biopsy, cloacal lavage, and large intestinal biopsy were performed on all 25 birds. Six of the birds were positive for influenza A RNA by RT-PCR. HA2 domain consensus primers were used to screen for H1 subtypes. Only a Brant goose (Branta bernicla) which was collected by G. D. Hanna on September 9, 1917 from the Pribilof Islands, AK was positive for an H1 Influenza A virus (A/Brant Goose/1/1917). The virus has been partially sequenced, including 500 nucleotides of the HA1 domain, 250 nucleotides of the nucleoprotein (NP), and 100 nucleotides of the matrix gene. From the preliminary data, it appears that all 41 antigenic site residues match the avian consensus as do the sites involved in receptor binding sites. Phylogenetically, the HA1 and the NP are near the root of the North American avian clade.

The current thinking is that there has been no significant drift in wild avian influenza A viruses in over 80 years and that the 1918 pandemic strain is phylogenetically distinct from the avian clade. Unlike the 1957 and 1968, the 1918 strain apparently did not acquire its HA directly from a bird strain but evolved for some time in a mammalian host. The 1918 NA also has more changes from avian sequences than the 1957 NA. Internal protein genes may or may not have re-assorted, although MA, NS, and NP gene sequences are consistent with retention from previously circulating human strains. Further studies of other avian influenza viruses circulating around the time of the 1918 pandemic as well as pre-1918 human influenza viruses are needed to fully understand the recently isolated 1918 virus.

3.2. A newly discovered human virus causing respiratory disease (Albert Osterhaus, Erasmus University, Rotterdam, The Netherlands)

A new virus, the human metapneumovirus (hMPV), has recently been characterized (van den Hoogen et al., 2001). The search for this virus began when several children presented with a history of RSV-like acute respiratory illness but had negative RSV test results. Cell culture showed an agent with slow replication in tMK cells and very poor growth in Vero and A549 cells. MDCK and CEF cells did not appear to support replication. Replication was trypsin-dependent and RSV-like cytopathic effect could be seen after 10–21 days of incubation. Paramyxovirus-like particles were seen on electron micrographs. No hemagglutination of turkey, chicken, or guinea pig erythrocytes could be demonstrated. As a result, the particles were screened by random priming polymerase chain reactions (PCRs) to clone and sequence the virus. Ten clones were recovered and indicated the virus to have similar genetic organization to avian pneumoviruses. Once sequencing of the first virus was complete, it was evident that the new virus was different from the avian pneumovirus. The virus would not replicate in the chickens, but did in the inoculated macaques. Two of four of the macaques had mild respiratory symptoms, predominately rhinitis. Microscopic examination of respiratory tissue found viral infection of the nose, lung, and alveoli 5 days

after infection. Based on these findings, the new virus was named the hMPV.

Next, serologic studies from children showed that antibodies to the hMPV were highly prevalent and that by 5 years of age all children investigated were seropositive. A collection of sera originally taken in 1958 was tested by immunofluorescence and virus neutralization assays. Antibodies were found in all the sera tested. Clinical data from samples collected prospectively during the 2000 winter season suggested that hMPV was associated with respiratory tract illness, particularly in young children and with a winter season predominance. Symptoms were similar to those caused by RSV with a range of illness from mild respiratory symptoms to severe bronchiolitis and pneumonia. Ten percent of children with a respiratory tract illness screened during this season had hMPV isolated without evidence of co-infection with other respiratory pathogens. Human MPV vRNA could not be detected in throat swabs from healthy children. Two genetic clusters of hMPV isolates were found in the Netherlands and may represent different serotypes of hMPV (clusters 99-1 and 00-1). The virus likely has a world-wide distribution and may have more significant impact in the very young and very old, in addition to patients with compromised immunity or underlying medical conditions. To date, antiviral susceptibility testing has not been evaluated on this virus.

3.3. Human metapneumovirus isolates in canada (Guy Boivin, Laval University, Québec City, Québec)

The hMPV is a newly described paramyxoviridae that is genotypically distinct from pneumoviruses and animal metapneumoviruses. Until recently, detailed descriptions of its epidemiology was limited to the Netherlands (van den Hoogen et al., 2001). It was decided to verify the presence of hMPV in Canada and describe its clinical spectrum. Thirty-nine specimens that had evidence of cytopathic effect on LLC-MK2 cells but not on MDCK, Hep-2, HFF, Vero, Mink lung, A-549, RD, 293, and Ht-29 cells, failed to hemabsorb with human O+ RBC, and had a negative RT-PCR for influenza A and B, RSV, adenovirus, parainfluenza virus 1-3, measles, and mumps were grown from respiratory samples of 38 patients from 1993 until 2001. Cytopathic effect was evident after a mean incubation time of 17.3 days (range 3–23 days). Only one isolate showed evidence of co-infections (measles), and rapid antigenic tests from samples were positive for concurrent influenza A or B in 13% and for RSV in 18%. Thirty-eight of thirty-nine (97%) isolates from 37 subjects were positive by RT-PCR for the hMPV F or M gene. Analysis of the F protein revealed a 94–100% similarity at amino acid level. Phylogenetic analysis revealed that all but one of the viruses clustered into one of two major groups. Electron microscopy revealed pleomorphic, spherical, and filamentous particles consistent with paramyxoviruses.

Clinically, 26 of the patients were hospitalized for febrile respiratory conditions, 7 were living in long-term care facilities, and 4 were seen in a private clinic. Eighty-seven percent (33/38) of samples were collected from December until May. The mean age was 45.5 (range 2 months–99 years). Most patients were younger than 5 years old (35%) or older than 65 years old (46%). Detailed clinical findings were obtained for 28 (75.7%) of the 37 patients. A third of the youngest patients had underlying conditions and presented with fever (92%), tachypnea or dyspnea (83%), cough (75%), and wheezing (50%). All of the patients were hospitalized. Three were admitted to the ICU, 2 required mechanical ventilation, and one patient with ALL died of pneumonitis and ARDS without another identified pathogen. Most of the older patients had underlying conditions and presented with cough (100%), fever (80%), and tachypnea or dyspnea (70%). Six of the elderly required hospitalization (the other 4 were in nursing homes) with 2 requiring ICU stays. Patients in the middle range typically presented with fever and cough (83%), tachypnea or dyspnea (50%), and sore throat (50%). Two-thirds had underlying conditions and 2 immunocompromised patients required hospitalization, one with mechanical ventilation in the ICU.

It is clear that a hMPV very similar to the virus described in Europe has circulated in Canada.

hMPV presents with a non-specific viral respiratory illness (VRI) typically at the extremes of age and in people with underlying medical conditions. It is able to cause a wide range of illnesses including mild upper respiratory symptoms and severe lower respiratory tract disease.

3.4. Thirty-two years of antigenic and genetic evolution in influenza H3 (Derek Smith, Erasmus University, Rotterdam, The Netherlands)

The hemagglutination inhibition (HI) assay is typically considered to have low resolution and to be capable of only reliably discriminating antigens when they have at least a 4-fold difference in HI titers. This resolution is sufficient to tell the difference between major drift variants, but is not sufficient for judging finer differences. A new mathematical method that can be used to make 'antigenic maps' from HI data has been developed. This new method increases the resolution at which HI can be interpreted and also provides a visualization of the antigenic relationships among many antigens. By merging existing HI tables, an HI table spanning H3 from 1968 to 2001, containing over 200 antigen strains and 60 sera, has been created. The antigenic map created from this merged table showed the patterns of antigenic evolution of H3 over the last 32 years. The map-making methods were also applied to genetic data producing a visualization of the genetic evolution of the 200 antigens in the merged HI data table, allowing a comparison of the antigenic and genetic evolution of influenza H3. This technique offers a new method of monitoring antigenic changes and evolution among influenza viruses.

4. Detection and surveillance

4.1. Detection of respiratory viruses (Maria Zambon, Public Health Laboratory Service, London, UK)

Diagnosis of respiratory viral infections is complicated by overlapping clinical spectra of infections, and multiple detection methods with variable sensitivity and specificity. Over the past 30 years diagnostic techniques have been refined and improved as the result of military research, space exploration requiring miniaturization, and a drive towards over-the-counter sales of treatments and diagnostics. The improvements have resulted in a reduction of the time to reporting of a positive result from well over a week to just a few hours. Currently available methods for detection of old and novel viruses include culture, immunofluorescence, near patient tests (NPTs), PCR, and serology. Each test has its own strengths and weaknesses and provides a useful role in overall diagnostic strategies.

Perhaps the greatest improvement in diagnostics has been the widespread availability of PCR. PCR has improved the detection of virus, aided in infection control, and assisted in appropriate prescribing. Unfortunately, it is an expensive technique that has also resulted in less culturing of virus with resultant loss of the antigenic information which is available only from viable virus. Several recent studies have shown a much higher rate of detection of respiratory viruses by PCR as compared to culture (Stockton et al., 1998; Carman et al., 2000; Fan et al., 1998). Parallel testing, such as is done with multiplex methods, is more efficient for detecting respiratory viruses rather than sequential serial testing for individual viruses, and ultimately labs will be running several different multiplex tests to complete a respiratory virus screen. PCR technology also allows for quantitation of virus (van Elden et al., 2001) as well as studies of molecular epidemiology. Increased sensitivity of diagnosis is particularly important in adults who shed less virus (e.g. RSV) and early in the infection, and it may also assist in surveillance (Zambon et al., 2001b). As a result of improved surveillance and sensitivity of molecular diagnosis, it has become clear that different respiratory viruses may co-circulate and there are occasions when acute respiratory infections during peak winter seasons are caused by more than one pathogen (dual infections). The clinical implications of co-circulation are still under investigation.

Use of quantitative real-time PCR is in its infancy for respiratory viruses, with the first few publications appearing for influenza. Although

the opportunities offered by such rapid diagnostics are very exciting, there remain several issues which require further research. These include the sensitivity of real-time PCR tests on respiratory samples coming from the community or from adults where the overall viral load is low, and the practical difficulties of selecting suitable detection combinations for multiplex analysis to avoid substantial parallel testing, and finding ways of adapting the chemistry of real time PCR to allow further analysis of products, e.g. sequencing/RFLP.

It is also clear that molecular approaches are essential for detecting novel pathogens. This has best been demonstrated with the recent description of hMPV. Human MPV was first described after using a random access PCR approach to test clinical samples and then subsequently refining primer sets to use in diagnosis (van den Hoogen et al., 2001). Newly developed PCR tests have been used to look at community respiratory samples collected during influenza season 2000–2001 to screen for hMPV in England. This season was a mild influenza season but hMPV was detected in 9 out of 400 influenza-like-illness (ILI) patients negative for influenza and RSV from England. From analysis of the clinical features of these cases, it appears that the virus presents as a non-specific respiratory viral infection with a peak incidence in December. The virus affects both sexes, all age groups (age 1–75), and was particularly common in elderly patients who had received the year's influenza vaccine.

Molecular archaeology involving PCR amplification from nucleic acids in archived tissue specimens is a powerful investigational tool. Such approaches have also been used to detect influenza from the 1918 pandemic and to allow further investigation of viruses in circulation at this time (Reid et al., 1999). In the past, it has been shown that the incidence of sudden infant death syndrome has correlated closely with the incidence of acute respiratory disease in the community. Preserved specimens from SIDS cases, and other appropriate pathological syndromes can now be screened for respiratory viruses.

Another major advance in diagnosis came in the form of office-based diagnostics or NPTs. These diagnostic kits vary in their complexity as well as their sensitivity and specificity. The Quidel Quickview dipstick Influenza test was analysed in vitro and in vivo. In an experimental virus dilution series, the rapid diagnostic test had a lower limit of detection of 1×10^{-4} dilution using the antigen detection whereas the kits were very much more sensitive if the nucleic acid was eluted from the strips and PCR used to detect viral genome. In field studies, this rapid diagnostic also performed less well than in did in the manufacturer's results (70–80% sensitivity vs. 40% sensitivity vs. PCR to 60% vs. culture). Taken together, these two results indicate the sensitivity limits of current technology for antigen antibody complexes. This simple method which involves detection of nucleic acid may be required to deliver the kind of sensitivity and specificity required for such tests to be truly useful for individual patient management. Along the same lines, methods to stabilize RNA, such as guanidinium in the transport media or swabs streaked onto filter paper imbedded with anti-degradation compounds, will enhance detection of viral genomes in clinical samples by PCR and enable easier and more broadly based sampling. Of note, these methods destroy virus infectivity and would require collection of a second sample for culture.

Serological diagnosis for respiratory viruses has depended on detection of antibodies in blood and the use of the hemagglutination inhibition tests (HI) tests for influenza and other viruses. However, there is increasing interest in the detection of antibodies in other body fluids, particularly in efforts to find non-invasive methods of sampling and alternative correlates of immune protection for vaccine trials. This is particularly relevant for the field of respiratory viral vaccines, where the mucosal delivery of vaccines is an important approach. It is therefore appropriate to consider the use of oral fluids as a source of antibody for development of alternative forms of antibody testing. There are several collection methods that may improve on the ability to collect gingival transudates (Nokes et al., 2001). These methods have been validated in the acute diagnosis of parvovirus, HIV, and hepatitisvirus infections as well as in the assessment of immunity and antibody

levels before and after measles, mumps, and rubella vaccination.

Array technology (macronucleotide, micronucleotide, and oligonucleotide chips) are emerging techniques that will, in the future, allow for diagnosis of infection as well as correlate disease pathogenesis with host response genes. The capacity to look for a broad array of genes will also allow for identification of genetic diversity. Genetic technology is also being used as part of sequence databases, as has been done with the influenza database described elsewhere in this article.

Finally, an emerging technique is the use of health forecast units (Dobson, 2001). It has been noted that changes in the weather correlate with health care utilization and increased mortality (Donaldson and Keatinge, 1997). As a result, units are being set up in England to forecast the probability of admissions to best guide the use of resources, based on meteorologic and other data. These units will collect maximum and minimum temperature, as well as weather patterns daily by postcode, socio-economic data by postcode, virology surveillance data, clinical morbidity, and hospital episode statistics by post code, age, length of stay, and diagnosis to produce predictions regarding probability of hospitalisation, allowing optimum use of health resources.

4.2. Emerging pathogens: the pandemic influenza threat from East Asia (Ken Shortridge, University of Hong Kong, Hong Kong SAR, China)

Examination of the patterns of occurrence of influenza pandemics over the last 1000 years has indicated the importance of China as their source, focusing on southern China in more recent years (Potter, 1998). This information has contributed to the hypothesis that southern China is a epicenter for the emergence of influenza pandemics (Shortridge and Stuart-Harris, 1982). This hypothesis traces its origins back to the domestication of the duck in China around 2500 BC. Once domesticated, the duck brought influenza viruses into the farmyard potentiating the opportunity to infect humans. A role for other domestic animals, such as the pig, for introducing non-human influenza viruses into the human population also appears likely (Scholtissek et al., 1985). Pandemic influenza, then, is a zoonosis, with domestic animals being the most likely immediate source of virus (Shortridge, 1992).

The understanding that southern China is a potential source of future pandemics provided a state of awareness by which the influenza A/H5N1 virus (H5N1/97) was recognized in chicken and humans in Hong Kong in 1997 (Subbarao et al., 1998; Claas et al., 1998; Yuen et al., 1998). The chicken was recognized as the source of the virus for humans. Influenza virus infections of chicken are uncommonly recognized but in 1997 in Hong Kong around 20% had detectable virus (Shortridge, 1999b). The chicken in this outbreak acted as an amplifying host for the H5N1 virus. The incident was stopped by the slaughter of all poultry across the Hong Kong (SAR) and a pandemic was believed to have been averted (Shortridge et al., 2000). The term 'averted' was used since it was not known if the highly pathogenic A/H5N1/97 virus occurred elsewhere in the region or if viruses that may have been its precursor did, too. The last human case was recognized the day before the slaughter commenced. It is a matter of speculation whether there would have been more human cases had the poultry not been slaughtered.

Reintroduction of poultry into the markets began in early 1998, at which time only ducks and geese among aquatic poultry were allowed into the single, segregated wholesale market. This segregation was put in place to minimize the spread of any viruses to land-based poultry which were sold live in the retail markets throughout the SAR (Shortridge, 1999b; Guan et al., 2000).

Intensive surveillance of poultry markets and antigenic and genetic analyses of isolates obtained in those surveillance studies and during the 1997 incident indicated that different virus precursors and hosts were involved in the genesis of the highly pathogenic H5N1/97 virus (Xu et al., 1999; Guan et al., 1999; Hoffmann et al., 2000). The precursor H5N1 virus from geese, i.e. Goose/Guangdong/96 (Go/Gd), acquired its replicating complex from a quail H9N2 virus, while the H6N1 virus, also from quail, provided the N gene

encoding the NA with a characteristic amino acid deletion in its stalk. This information engendered a precautionary principle which enabled Hong Kong to monitor poultry in various settings in order to limit the risk of an H5N1/97-like virus reemerging or regenerating locally, not only as an economic threat to the poultry industry but, more importantly, as a potential global health threat to humans (Shortridge, 2001).

Another potentially dangerous H5N1 virus was recognized in Hong Kong in May 2001. During 1999, the H5N1 Go/Gd virus was isolated from geese and in mid-2000, it was detected in ducks. Later in 2000, the virus was found to have undergone re-assortment with an unknown avian influenza virus from waterfowl infecting ducks and geese in the wholesale market. By April 2001, five genotypes of precursor Go/Gd H5N1 were isolated from apparently healthy chickens in retail markets. On genetic grounds, it was predicted that they would be highly pathogenic for chicken, which was confirmed in experimental pathogenicity studies (R.G. Webster, personal communication). To address the impending threat, the Government's Expert Working Group met to develop unified strategies to deal with the markets, media and public relations in the event that chicken were to die in the retail markets. Indeed, in mid-May 2001, chicken infected with the Go/Gd H5N1 genotypes began to die. Soon after, the decision was made to slaughter all poultry across the SAR, except for chickens on certain farms in the New Territories over a defined age. This decision was taken to minimize the risk of the five genotypes in chicken re-assorting with the H9N2 and the H6N1 viruses present in quail in the live retail markets to produce a virulent H5N1/97-like virus. The slaughter was carried out in a phased operation, and no human case was recognized.

The introduction of a rest day once a month in which all poultry in all markets must be sold or, if not, killed, and the markets thoroughly cleansed and disinfected, is a step toward preventing the amplification of viruses in the markets. In the event that a H5N1 Go/Gd-like virus might still gain entry into the retail markets, the planned withdrawal of the quail, which hosts H9N2 and H6N1 viruses, from all markets is seen as a risk minimization step for the generation of an H5N1/97-like virus.

Hong Kong, then, is an influenza sentinel post for the wider region (Shortridge et al., 2001). While Hong Kong may be able to react quickly to an influenza incident, it cannot react for areas beyond its geographic confines. Based on surveillance studies on poultry, the viral genes and their hosts are still present in the immediate area and could allow a H5N1/97-like virus to reappear or, alternatively, some other re-assortant of it to appear. The five Go/Gd H5N1-like viruses of 2001 probably represent an 'intermediate' or 'transitional' virus not detected in 1997. Pre-emptive slaughter in 2001 probably prevented its progression to a virulent human pathogen beyond this hypothetical stage. Duck raising practices similar to those in southern China are carried out in many parts of East Asia, which suggests the possibility that an H5N1/97-like virus could arise elsewhere within this wide region. In chicken, an H5N1 virus could be introduced into a new setting or area under cover of the heterosubtypic immunity afforded by a previous H9N2 infection (O'Neill et al., 2001). However, while H9N2 viruses may occur in the region, the occurrence of the H5N1 Go/Gd-like virus and the H6N1 virus with the characteristic NA in poultry is unknown. Moreover, unless the H5N1 Go/Gd virus occurs beyond east Asia, it is presently hard to imagine the pathogenic form arising in other regions. The H9N2 virus in its own right is a cause for concern as the genes of its replicating complex are similar to those of the H5N1/97 virus, (Guan et al., 1999) and one lineage of the virus has already been isolated from two young children with influenza-like illness in Hong Kong (Peiris et al., 1999; Lin et al., 2000) and another from pigs imported into Hong Kong (Peiris et al., 2001). The H9N2 virus has now been recognized in poultry beyond East Asia in the Middle East, Europe, and Southern Africa and could be a cause for concern both inside and outside the epicenter (Alexander, 2001; Cameron et al., 2000).

History usually repeats itself and insofar as the origins of pandemics are concerned, attention must be paid to China, particularly southern China, for the early detection of a future pan-

demic. The geographical area of southern China has been the origin of the 1957 H2N2 and 1968 H3N2 pandemics, the H5N1 incident of 1997 and, as outlined here, the H5N1 event of 2001. Furthermore, there is circumstantial evidence for it being the origin of the 1918 H1N1 pandemic (Shortridge, 1999a). Should a pre-pandemic or pandemic virus smolder in a human or non-human host, it could eventually emerge in East Asia outside the epicenter or it could indigenously arise there. Furthermore, given the complex population dynamics of humans and domestic poultry in recent times, the possibility that the next pandemic could arise even beyond East Asia cannot be excluded.

5. Pathogenesis and immunology

5.1. Rhinovirus and the lower airways: mechanisms of disease and implications for treatment (Sebastian Johnston, National Heart and Lung Institute, Imperial College of Science, Technology, and Medicine, London, UK)

Several epidemiologic studies have linked rhinoviruses with exacerbation of asthma and chronic obstructive pulmonary disease (COPD). More recently the mechanisms of rhinovirus induced asthma have begun to be understood.

In a large study of 114 children with wheezing or cough in the past year, children were asked to keep a daily diary of symptoms and see a physician for evaluation if they had an asthma exacerbation, upper or lower respiratory tract symptoms score $\geqslant 4$, or a subjective feeling of having a cold. Routine cultures and PCR for rhinoviruses and coronaviruses testing on collected samples found respiratory virus infections, most commonly HRV, in 80–85% of patients with asthma exacerbations (Johnston et al., 1995). A number of more recent studies have confirmed these findings (Rakes et al., 1999; Freymuth et al., 1999).

Adults with COPD often develop exacerbations of their underlying disease when they are infected with rhinoviruses. In a study of 83 patients with moderate to severe COPD, nasal aspirate and/or nasopharyngeal swab was collected during 168 exacerbations. PCR was done for all common respiratory pathogens and serum was collected for inflammatory markers. Thirty-nine percent of exacerbations were associated with virus detection, 58% of which were rhinoviruses. Viral exacerbations were associated significantly with increased dyspnea, higher total symptom score at presentation, longer median duration of symptoms, greater falls in peak expiratory flow rates, and higher plasma levels of fibrinogen and IL-6 (Seemungal et al., 2000).

A crucial question about the pathogenesis of rhinovirus induced lower airway disease is whether the virus directly infects the respiratory epithelium leading to lower airway inflammation or if the inflammation is the consequence of neural reflexes or systemic immune responses to infection restricted to the upper respiratory tract. It is now clear that the virus can replicate successfully at 37 °C and that certain strains may grow better at body temperature, as would be found in the lungs, than at cooler temperatures found in the upper respiratory tract (Papadopoulos et al., 1999). Additionally, rhinoviruses replicate in vitro in primary bronchial epithelial cell cultures as evidenced by increasing viral titers, increasing viral RNA, new viral protein synthesis, and visible cytopathic effect (Papadopoulos et al., 2001). In clinical samples from experimentally infected volunteers, in situ hybridization of biopsy specimens detected rhinovirus genomic and replicative RNA strands at the same detection rate (50%) as found in upper respiratory tracts samples (Papadopoulos et al., 1999). Infection of these cells in vitro also causes the release of proinflammatory mediators like RANTES, (Schroth et al., 1999) IL-6, IL-8, IL-16, (Papadopoulos et al., 1999) and upregulation of cell surface markers like major histocompatibility complex (MHC) class I and B7.2, (Papi et al., 2000) ICAM-1, (Papi and Johnston, 1999b) and VCAM-1 (Papi and Johnston, 1999a). Natural and experimental infections are also associated with evidence of inflammation as shown by increases in mucosal T cells, eosinophils, (Fraenkel et al., 1995) mucosal mast cells, macrophages, and in sputum eosinophilic cationic protein, IL-6, IL-8, (Grunberg et al., 2002) and ICAM-1 (Grunberg et al., 1997). There is also emerging evidence that

leukotriene levels and the leukotriene and prostanoid synthetic enzymes 5-lipoxygenase, FLAP and cyclooxygenase (COX)-2, are elevated after rhinoviral infection and the degree of elevation correlates with severity of symptoms (Seymour et al., in press). Finally, there is evidence of cellular damage with elevated levels of sputum LDH in patients with asthma exacerbations secondary to RSV, influenza, and rhinoviruses. The degree of LDH elevation also appears to be a major predictor of length of hospital stay and therefore likely is a marker of severity of infection (Wark et al., in press).

The host response may play a role in the exacerbation in other ways. Peripheral blood mononuclear cells from asthmatics that are incubated with rhinoviruses in vitro have deficient IFN-γ and IL-12 responses relative to non-asthmatics, suggesting that deficient type 1 immune responses may increase susceptibility of asthmatic subjects to respiratory viral infection (Papadopoulos et al., in press). In clinical studies of asthmatic versus non-asthmatics, this altered immune response does not appear to increase the frequency of infections nor does it seem to modulate the duration or severity of upper respiratory tract disease. Asthmatic patients do, though, have more severe and prolonged lower tract disease and more severe fall in their peak expiratory flow rates (Corne et al., 2002).

Rhinoviruses are thus a major cause of both asthma and COPD exacerbations. Direct lower airway infection and resulting inflammation appear to be the major mechanism of these exacerbations, although asthmatics may have a deficient type 1 immune response that contributes to the problem. As a result, therapy for rhinovirus induced lower respiratory tract disease should ideally reach the lower airways.

5.2. Viral infections and allergic disease (Larry Anderson)

Viral infections have been hypothesized to both predispose to and protect from later development of asthmatic disease, as well as acutely precipitate asthmatic attacks in persons with asthma. RSV has been of particular interest in relationship to asthmatic disease because it causes obstructive airway disease with wheezing that is clinically similar to asthma. In addition, RSV lower respiratory tract disease in infancy has been associated with childhood asthmatic disease possibly because underlying asthmatic disease predisposes to RSV disease or possibly because RSV infection predisposes to asthmatic disease. This link between RSV disease and asthma raises the possibility of parallels in the pathogenesis of the two diseases. RSV is the single most important cause of serious lower respiratory tract illness in infants and young children and causes repeated infections throughout life that can be associated with serious LRI especially in patients with compromised cardiac, respiratory or immune systems and the elderly. The similarities between the clinical and some pathogenic features of RSV and asthmatic disease suggest that lessons learned from one may provide clues to pathogenesis of the other.

RSV is a negative stranded RSV virus and its genome encodes 11 proteins including two surface proteins important for inducing protective immunity, the fusion (F) protein and the putative binding (G) protein. The F protein is most effective in inducing protective immunity. The G protein induces some degree of protective immunity but also appears to participate in the disease process. We have recently identified two factors that contribute to the biology of RSV infection and probably pathogenesis of disease, and both are associated with the G protein. These two factors could also participate in the pathogenesis of asthmatic disease. First, the RSV G protein contains a chemokine motif, CX3C, and the G protein has properties similar to the corresponding CX3C chemokine, fractalkine (Fkn). Second, RSV, possibly through the G protein, induces the neurokinin, substance P (SP), and induction of SP is associated with an increased inflammatory response in the lung of RSV-infected mice.

Both the RSV G protein and Fkn are large, highly glycosylated gylcoproteins that contain intracellular, transmembrane, and extracellular domains; both have heparin binding domains (HBDs); both have the CX3C motif; and both produce a transmembrane and a shorter secreted form. The G protein also has functional proper-

ties similar to Fkn. It binds to the CX3C chemokine receptor, CX3CR1, as shown in binding and binding inhibition studies using 293 cells transfected with the CX3CR1 (293-CX3CR1). The percent of cells positive for G protein binding increase from ~15% in 293 cells to ~80% in 293-CX3CR1 transfected cells, and this increased binding was inhibited by G protein monoclonal antibodies, Fkn, anti-CX3CR1 antibodies, and a G protein peptide containing the CX3CR motif. G also blocked Fkn binding to 293 CX3CR1 cells. Interestingly, RSV binding to CX3CR1 facilitates infection of cells as indicated by plaque reduction assays. Since RSV also uses HBDs to infect cells, we first blocked the HBDs with heparin. Heparin gave a 66% reduction in plaques but the addition of G protein, G protein peptide with the CX3C motif (and not peptides without the CX3C motif), Fkn, or anti-CX3CR1 antibodies increased the reduction in RSV plaques to >90%. Finally, G protein, through the CX3C motif, induces leukocyte chemotaxis in a fashion similar to Fkn. We used modified Boyden chambers to study leukocyte chemotaxis and found that ~4-fold more cells migrated toward the G protein or Fkn than the media control. Fkn, anti-G but not anti-F protein monoclonal antibodies, and anti-CX3CR1 antibodies inhibited cell migration toward G. In addition, cloned expressed G protein that contained the CX3C motif induced leukocyte migration but G protein that lacked this motif did not induce leukocyte chemotaxis. Thus, the CX3C motif in the G protein appears to be important to the biology of RSV infection and possibly pathogenesis of disease. This region of the G protein could be important to development of both vaccines and anti-viral drugs.

The CX3C motif on G may also participate in asthmatic-like aspects of RSV disease as suggested by the ability of Fkn to induce SP. SP is a neurokinin that has linked to both asthmatic disease and RSV disease. SP is an 11 amino acid tachykinin that has a range of effects that include induction of vasodilation, increased vascular permeability, mucus secretion, and induction of immune and inflammatory responses. RSV infection increases levels of SP in bronchial alveolar lavage (BAL) specimens in mice and addition of SP to immune activated lymphocytes increases proliferation. When we inhibited the action of SP with anti-SP antibodies, the inflammatory response to RSV infection was decreased. This is particularly evident in studies of mice immunized with formalin-inactivated (FI) RSV vaccine. FI-RSV immunized mice mount an enhanced inflammatory response to RSV challenge. Treatment of FI-RSV-immunized mice with anti-SP antibodies blocked much of this enhanced inflammatory response as exemplified by the decrease in percent of eosinophils from ~30% to ~10% and polymorphonuclear cells from ~18% to <1% at 2 days post challenge in BAL samples. The ability of anti-SP antibodies to decrease the inflammatory response to RSV infection suggests that these antibodies might be useful as an adjunct to antiviral treatment. The fact that anti-viral drugs and neutralizing antibodies or monoclonal antibodies are effective at decreasing viral titer but relatively ineffective at decreasing disease, raises that possibility that a substantial proportion of RSV disease results from virus-induced immune and inflammatory responses. Steroids have been considered as a means to decrease the RSV-associated inflammatory response but steriods also delay virus clearance. We found that addition of anti-SP antibodies to treatment with an RSV neutralizing F protein antibody before 2 or 6 days after RSV challenge markedly decreased the inflammatory response and did not delay virus clearance. For example, the total number of cells was decreased ~2-fold within one day of administration of anti-SP antibodies. Anti-SP antibodies (or other means to block SP activity) could be an effective adjunct to anti-RSV drug or immune therapy.

5.3. Immunopathogenesis of vaccine-enhanced RSV disease (Peter Openshaw, Imperial College of Science, Technology, and Medicine, London, UK)

Although immune responses to virus infections are usually protective, they can also be harmful (Openshaw et al., 2001). Inducing a strong immune response is an essential aim of vaccination, but risks disease enhancement. In infantile bronchiolitis, the most severe disease typically develops

several days after the apparent onset of infection, at a time when viral titres are low or falling but inflammatory responses are at a peak. Animal models strongly suggest that vaccine enhancement of RSV disease may be due to strong and perhaps unbalanced T cell priming, although infection-enhancing antibody may also play a role in enhanced inflammation.

In mice, enhanced disease can result from over-exuberant CD8 + cells which cause extensive pulmonary necrosis and an acute capillary leak-like syndrome, similar to ARDS or CD4 cells which recruit an abundant cellular infiltrate, the nature of which is dependant on the cytokine and chemokine patterns of memory T cells. Formalin vaccination has been linked to the induction of 'Th2 cells', which make IL-4 and IL-5 and induce a strong pulmonary eosinophilic response. Possible immunotherapeutics for enhanced disease include: (1) T cell depleting agents, particularly those specific for disease-enhancing subsets; (2) cytokine blockers (e.g. anti-TNF); and (3) chemokine blockers, particularly those for RANTES and MCP-1.

Prior exposure of BALB/c mice to the attachment (G) or fusion (F) protein of RSV increases illness severity during intranasal RSV challenge, due to Th2-driven lung eosinophilia and exuberant Th1-driven pulmonary infiltration respectively. We and others have used this model to test potential vaccines and immunomodulators. For example, we have tested a detoxified mucosal adjuvant derived from the labile toxin of *Escherichia coli*, LTK63. When mixed with a nine amino acid second matrix protein peptide (M2 62-91) and given intranasally, it is capable of inducing 'protective' cytotoxic T cells (CTL) which reduce lung virus. Unfortunately, such CTL did not ameliorate disease, and in fact made it worse. A corollary observation in this model was that signs of illness were reduced by anti-INF-γ antibody therapy (Simmons et al., 2001). To give a second example, we have tested anti-TNF treatment in mice with 'shock lung' induced by CTL or Th1 cells and the eosinophilic disease induced by Th2 cells; in either case, it was beneficial (Hussell et al., 2001). By contrast, anti-T1/ST2, a surface ligand expressed on Th2 but not Th1 cells, only reduces lung inflammation and severity of illness in mice with Th2-driven immunopathology (Walzl et al., 2001).

Recent studies established that the chemokines RANTES and MCP-1 appear important. RANTES and MCP-1 mRNA and protein levels are elevated in RSV infection, particularly the eosinophilic Th2 augmented response. Preliminary blocking studies using metRANTES show reduced infiltration of CD4 + and CD8 + cells with increased viral replication; other blocking studies are underway. An additional area of interest is the role of eotaxin in RSV infection, since this chemokine appears to play a major role in the polarization of response between the Th1 and Th2 dominated diseases. Unfortunately, experimental models have failed to find a difference in eotaxin expression in any particular form of RSV disease, raising the possibility that other eosinophil chemoattractants are involved.

From this data, it seems that immune response to RSV in man is likely a balance between virus elimination and immune pathology. Our current understanding of the pathogenesis of bronchiolitis supports the concept that combined therapy with antivirals to control viral replication and immunomodulators to control the exuberant immune response would be a rational treatment approach to this complex disease.

5.4. Viral otitis media (Terho Heikkinen, University of Turku, Finland)

Although AOM is generally considered a bacterial infection, about one-third of AOM cases remain without a proven bacterial etiology (Heikkinen, 2000). The vast clinical experience connecting AOM with viral upper respiratory tract infections (URIs) has prompted investigators to search for the role of viruses in the etiology of AOM, and there is now convincing evidence to establish the crucial role of respiratory viruses in the etiopathogenesis of AOM.

The role of viruses in otitis media is not limited to their presence in the MEE (middle ear effusion) during AOM. In fact, viruses could have a crucial role in the development of AOM even if they never enter the middle ear cavity itself. Both

clinical experience and extensive studies have demonstrated the tight association between viral upper respiratory infections and AOM (Henderson et al., 1982; Ruuskanen et al., 1989; Arola et al., 1990a). Studies of the temporal development of AOM in young children have shown that the peak incidence of AOM is on days 3–4 after the onset of symptoms of URI (Heikkinen and Ruuskanen, 1994; Koivunen et al., 1999). Therefore, in the vast majority of cases in children, AOM can be clearly regarded as a complication of URI.

Direct evidence for the role of respiratory viruses in the pathogenesis of AOM has been obtained by detaching for them in MEE of children with AOM. Since the 1950s, investigators have attempted to isolate viruses from MEEs. Although data from a few studies performed mainly during influenza or RSV outbreaks demonstrated isolation of viruses from the MEE, (Yoshie, 1955; Berglund et al., 1966, 1967) the overall detection rate of viruses during the 1950s and 1960s remained lower than 5%. Since the 1980s, the improved viral culture techniques and the development of viral antigen detection methods have allowed demonstration of viruses or viral antigens in approximately 20% of children with AOM (Chonmaitree and Heikkinen, 1997). In about two-thirds of cases when virus has been found in MEE, bacteria have also been isolated, indicating a mixed infection. With the use of viral culture and antigen detection methods, virus as the only pathogen in MEE has been detected in 6% of AOM cases.

The development of the PCR technique has substantially increased the rates of viral detection in MEE. In recent PCR-based studies, viral materials have been detected in up to 70% of MEEs from children with AOM (Okamoto et al., 1993; Pitkäranta et al., 1998; Chonmaitree and Henrickson, 2000). Obviously these high rates of viral detection have raised the question whether viral nucleic acids detected by PCR represent viruses with a real pathogenetic role in the middle ear, or whether they represent viral materials that have migrated passively from the nasopharynx to the middle ear along with nasal secretions.

Recent data indicate that there are differences between various respiratory viruses in their ability to invade the middle ear. In a study of 456 children with AOM, (Heikkinen et al., 1999) the relative rate of middle-ear invasion by RSV was significantly higher than the rates by any of the other viruses searched for. Also, parainfluenza viruses and influenza viruses were found to invade the middle ear significantly more often than enteroviruses or adenoviruses. A recent PCR-based study (Pitkäranta et al., 1998) suggested that the relative prevalence of rhinovirus RNA in MEE during AOM may be similar to that of RSV. These results suggest that while some viruses may enter the middle ear passively along with nasal secretions, whereas other viruses may actively invade the middle ear and significantly contribute to the inflammatory process in the middle ear cavity.

The presence of viruses in the MEE is important not only in regard to the etiology and pathogenesis of AOM, but viruses may also have a profound impact on the outcome of the disease. In a study of 58 children with AOM, (Chonmaitree et al., 1990) MEEs were obtained before and 2–4 days after initiation of antibiotic treatment. At the latter visit, even though the etiologic bacteria were susceptible to the antibiotic used, bacteriologic failure was observed in 33% of the children who had both bacteria and virus in the initial MEE, compared with 3% of children with only bacteria in the MEE. In another study of 22 children with AOM unresponsive to 48 h of antibiotic treatment, (Arola et al., 1990b) viruses were recovered from MEEs of 32% of these children, compared with 15% of children in a comparison group with newly diagnosed, untreated AOM. The detailed mechanisms by which viruses may enhance or prolong the inflammation in the middle ear remain to be determined, but some studies have shown higher concentrations of inflammatory mediators in MEEs containing both bacteria and virus than in those containing bacteria alone (Chonmaitree et al., 1991, 1994).

Despite the well-established correlation between viral infection and AOM, the rates of detection of respiratory viruses in nasopharyngeal specimens from children with AOM have conventionally ranged between 30 and 50% (Ruuskanen and Heikkinen, 1994). It is obvious, however, that limitations of viral culture and antigen detection

methods have resulted in underdetection of viruses in nasopharyngeal specimens. In recent studies utilizing PCR, respiratory viruses have been found in the nasopharyngeal specimens of up to 90% of children with AOM (Ruuskanen and Heikkinen, 1994; Heikkinen et al., 2001).

With respect to prevention of AOM, there are two distinct stages during the pathogenesis of this disease where potential intervention could be instituted. First, the whole preceding viral infection could be prevented by viral vaccines, which would also prevent the development of AOM as a complication. Second, during the first few days after the onset of respiratory symptoms, antiviral drugs could be used to provide sufficient attenuation of the viral infection so that the development of AOM could be prevented. Clinical studies have demonstrated that both of these approaches are effective. Several influenza vaccine trials have shown that prevention of the underlying viral infection is an effective way to prevent the development of AOM, (Heikkinen et al., 1991b; Clements et al., 1995b; Belshe et al., 1998) and it is obvious that vaccines against other major viruses would also be effective. Further, a recent study in children with influenza indicated that the initiation of oseltamivir treatment during the first 48 h after the onset of clinical symptoms resulted in a 44% reduction in the development of AOM (Whitley et al., 2001).

In summary, viral infection plays a key role in otitis media because it initiates the whole cascade of events that finally leads to development of AOM. At least some viruses seem to be active in invading the middle ear cavity, where viral–bacterial interaction may result in enhanced inflammation and delayed resolution of the disease. Both prevention of the preceding viral infection by vaccines and attenuation of the viral disease by antiviral agents would be effective in the prevention of AOM.

5.5. Cytotoxic T lymphocyte response to respiratory viral infections (Guus Rimmelzwaan, Erasmus University, Rotterdam, The Netherlands)

CD8+ cytotoxic T lymphocytes (CTL) contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. This recognition by specific CTL may lead to the elimination of infected cells. Some viruses have developed strategies to evade recognition by CTL. One of these strategies involves antigenic variation in CTL epitopes and has been described for viruses, which chronically infect their host like EBV, HIV HBV and HCV. Influenza viruses, which cause acute respiratory infections in a significant portion on the human population annually, can also escape from CTL immunity by mutations in CTL epitopes. Three variable CTL epitopes in the influenza virus NP have recently been described.

The first two examples involve a mutation at position 384 of the NP, R384G, which is the anchor residue of an HLA-B27 restricted epitope $NP_{383-391}$ (SRYWAIRTR) and an HLA-B8 restricted epitope $NP_{380-388}$ (ELRSRYWAI). Previous studies have shown that these mutations have arisen in the 1993/1994 influenza season and that these mutant variants completely replaced the virus strains containing the wild type epitopes. Furthermore it was shown using T cell clones specific for both epitopes that T cell recognition was completely abrogated by the R384G mutation. This was the first evidence that influenza viruses can escape from surveillance by specific CTL (Voeten et al., 2000).

The third example of variation in an influenza virus CTL epitope associated with escape from CTL was found in a newly identified HLA-B35 restricted CTL epitope in the NP (Boon et al., in press). After defining the minimal epitope $NP_{418-426}$ using T cell clones, an epitope prediction program (http://www.umds.ac.uk/tissue) and synthetic peptides, it was found that this immunodominant epitope exhibited extensive amino acid sequence variation and that the variants emerged in a chronological order. All variants studied had similar binding affinities for the HLA-B35 molecule, suggesting that they all represented CTL epitopes. Using T cell clones and polyclonal T cell preparations directed against previous and current variants of the epitope, the recognition of these epitopes was studied. Again CTL specific for older variants failed to recognize more recent strains of influenza A virus, indicating an escape from CTL immunity.

Thus, in addition to mutations in surface glycoproteins like the HA allowing escape from antibody mediated immunity, the newly described mutations in the viral NP suggest that influenza viruses can also escape from CTL mediated immunity.

5.6. HLA associations of influenza vaccine non-responsiveness (Colin Gelder, University of Wales, Cardiff, UK)

A pilot study looked at HLA class II associations of influenza vaccine non-responsiveness in a cohort of 73 at risk individuals recruited from a single family practice in England (Gelder et al., 2002). Donors were molecularly HLA class II typed, and hemagglutination inhibition (HAI) titers were measured before and 28 days after subunit vaccination. Non-responsiveness was defined as failure to mount an HAI response to any component of the trivalent influenza vaccine. An association between failure to mount a HAI response to influenza vaccine and the DRB1-*0701 locus was found (13/32 non-responders versus 6/41 responders, $P = 0.016$). The mechanisms underlying this phenomenon are unclear though as the DR7 locus has been associated with poor response to hepatitis B vaccine, another highly purified subunit vaccine, it is proposed that some DRB1-*0701 donors may have a defect in T cell help for antibody production. This is the first report that polymorphisms in HLA class II molecules modulate antibody responses to influenza vaccination. The work needs to be confirmed in other populations.

5.7. Impact of age and COPD on $CD8^+$ CTL responses to influenza and RSV (Innocent Mbawuike, Baylor College of Medicine, Houston, TX)

Elderly persons represent a high-risk group for severe influenza disease, pneumonia and death. The Centers for Disease Control estimates that of the approximately 20 000–40 000 annual influenza epidemic-associated deaths, 80–90% occur among persons >65 years of age (ACIP, 2001). Recent studies indicate that RSV is also a major cause of morbidity and mortality among elderly persons (Zambon et al., 2001b,b; Falsey, 1998; Falsey and Walsh, 1998; Falsey et al., 1995; Drinka et al., 1999a,b; Fleming and Cross, 1993). Exacerbation of COPD is a major cause of morbidity and mortality and hospitalization (Glezen et al., 2000; Greenberg et al., 2000). Influenza and RSV infections have been identified as major risk factors for COPD exacerbations (Walsh et al., 1999; Greenberg et al., 2000; Glezen et al., 2000; Falsey and Walsh, 2000). MHC class I-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) activity is thought to play a major role in the recovery from influenza and RSV infections and disease (McMichael et al., 1983; McMichael, 1999).

Recently, we designed a study to investigate whether $CD8^+$ CTL activity against influenza A and influenza B viruses and RSV is diminished in elderly compared to young adults, to assesses the impact of COPD on CTL responses to these viruses, and to identify the surrogates of CTL responses in these two high risk groups. Peripheral blood lymphocytes (PBL) were obtained from elderly COPD subjects and a control cohort matched for sex, age and other parameters during four respiratory virus epidemic periods (1991–1995). Pre-season (September–October), in-season (November–January) and post-season (March–May) PBL samples were cryopreserved and stored in liquid N_2. Virological and serological tests identified a total of 33 subjects (mean age, 68 ± 2) as being infected with influenza A/H3N2, A/H1N1, influenza B or RSV. Nine of these were COPD patients while 24 were control subjects. Cells were thawed and stimulated in vitro with RSV, influenza A/Taiwan/1/86 (H1N1), A/Beijing/353/89 (H3N2) and influenza B Panama virus. The level of $CD8^+$ CTL lysis of autologous target cells infected with RSV or influenza virus was determined in a 4-h ^{51}chromium release assay. The levels of IFN-γ and IL-4 in CTL culture supernatants were measured using ELISA. Cryopreserved cells from 6 healthy adults (mean age, 34 ± 5 years) were included as controls during each assay run (2–7 different occasions). Elderly study subjects exhibited significantly lower CTL responses to influenza A/H3N2, A/H1N1, influenza B and RSV than young adult controls. CTL

responses to influenza A/H1N1 and A/H3N2 viruses were somewhat higher among COPD than non-COPD individuals infected with the same viruses. Also, a slightly lower RSV-specific CTL response was displayed by subjects with COPD than normals. IFN-γ, a surrogate marker for $CD8^+$ CTL activity, and IL-4, a CTL antagonist, were produced at similar levels by COPD and normal subjects. In contrast, elderly persons produced significantly lower IFN-γ and somewhat higher levels of IL-4 compared to young persons.

These results demonstrate that among elderly COPD patients, $CD8^+$ CTL responses to influenza viruses and RSV are not impacted significantly by COPD, while aging diminishes these responses. Reductions in IFN-γ production among elderly persons suggest that type 1 immune deficiency may contribute to $CD8^+$ CTL deficit among elderly persons. The role of $CD8^+$ CTL deficiency in increased morbidity and mortality among elderly persons infected with respiratory viruses requires further evaluation.

5.8. Prenatal viral infection in mice leads to pyramidal cell atrophy and macroencephaly in adulthood (S. Hossein Fatemi, University of Minnesota, Minneapolis, MN)

Schizophrenia and autism are neurodevelopmental disorders wtih genetic etiologies as demonstrated by a monozygotic twin concordanance rates of 46 and 64%, respectively. Many have hypothesized that there are environmental exposures that predispose to these conditions. Since there is an increased risk of developing schizophrenia and autism in the offspring of mothers who were exposed to viral infections during pregnancy, a study was conducted to assess the hypothesis that intranasal infection of day 9 pregnant C57Bl/6 mice to H1N1 A/NWS/33 would cause structural abnormalities in the exposed progeny. Neonatal brains from day 1, 14, 35, and 56 were analyzed for reelin, SNAP-25, neuronal nitric oxide synthase (nNOS), and GRAP by immunohistochemistry and western blot. Brains were also microscopically reviewed for morphometric analysis and cell counts. Reelin is an extracellular protein has been described to be deficient in patients with schizophrenia, bipolar affective disorder, depression, lissencephaly, and autism. It was found in lower levels in neonatal mice of infected mothers at day 1 as compared to offspring of unexposed mothers (Fatemi, 2001; Fatemi et al., 2000b,c, 2001b). SNAP-25, a presynaptic membrane protein, is decreased in the hippocampus of schizophrenic patients. In the mice, it was found to be higher at day 1 in offspring born to infected compared to uninfected mothers (Fatemi et al., 2001a). nNOS expression is abnormal in schizophrenic brains. In the neonatal mice, there was increase activity in the rostral areas of the brain at day 14 and 35 with decreased activity at day 56 in the exposed vs. unexposed mice (Fatemi et al., 2000a). GFAP, a filamentous protein of the astrocyte, is a marker of cellular injury and was found to be elevated at days 1, 14, and 35 in offspring of infected mice. Morphologically, there was thinning of the hippocampus and neocortex in day 1 exposed neonates. Pyramidal cell density increased while non-pyramidal cell density decreased at day 1, although both densities were elevated by 14 weeks in exposed brains. There was persistent pyramidal cell atrophy from birth to adulthood and evidence of macrocephaly and decreased ventricle size in adult exposed mice. Finally, there was no evidence of viral protein within the brains of the neonatal mice (Fatemi et al., in press). Whether any of these findings are specific for influenza infection of the pregnant mothers are unclear.

5.9. A schizophrenia model: maternal influenza infection alters fetal brain deveopment (Paul Patterson, Caltech, Pasadena, CA)

Another model was developed to test the hypothesis that offspring of influenza infected mothers displayed schizophrenic behavior. The primary approaches are neuropathology and behavior, and there are several behavioral assays in which animals and humans can be directly compared, (Lipska and Weinberger, 2000) including anxiety in novel situations and the acoustic startle response, which has repeatedly been found to be abnormal in schizophrenic (Geyer et al., 1999) and recently in autistic individuals, and is thought to reflect difficulties in sensimotor gating.

Pregnant BALB/c and C57/Bl6 mice were infected on day 9 of pregnancy with a sublethal dose of mouse-adapted human influenza virus (A/NWS/H1N1). Behavioral studies were carried out on the adult offspring. In the open field test, mice are individually placed near the center of the box and their movements followed by videotaping over a 10 min period. The novel object test was carried out immediately after the open field test; the object was a round silver cup. Social interaction between pairs of mice of the same experimental and control groups was also examined by placing two mice of the same sex who had been housed separately in the center of the box, approximately 10 cm apart, and monitoring their contacts.

In all of these tests, the offspring of infected mothers displayed behaviors consistent with human schizophrenia and/or autism. Exposed mice were less likely to explore their environment and spent 8-fold less time in the center squares of the open field, entered the center squares nearly 6-fold less often, and they explored their environment by rearing on their hind legs 4-fold less often than control mice. The mice born to infected mothers also had an almost 2-fold greater latency in first contacting the novel object, and they initiated almost 3-fold fewer contacts with the object than mice born to sham infected mothers. In the social interaction test, the infected mice contacted each other 2.7-fold less frequently than the mice born to sham infected mothers.

The acoustic startle response was assessed in an automated startle chamber (Swerdlow and Geyer, 1998; Koch, 1999). When a prepulse, too small to cause a startle itself, precedes the startle stimulus, the response is diminished, a phenomenon that is termed prepulse inhibition (PPI). PPI in control offspring caused a 50–54% inhibition of the startle response while response in the exposed mice fell into two groups: 59% had similar responses to the controls while the remaining 41% displayed a striking deficit in PPI. This is consistent with PPI findings in schizophrenic and autistic subjects.

The mice born to infected mothers were also compared to controls in their response antipsychotic and psychomimetic drugs using the PPI assay. Previous experiments showed that acute administration of the dopamine receptor blocking, antipsychotic drugs clozapine and chlorpromazine increase PPI in rodents (Swerdlow et al., 1998). This response was dramatically accentuated in the mice born to infected mothers, suggesting an abnormal dopamine system. In the converse experiment, ketamine, a glutamate receptor blocking, psychomimetic drug, which exacerbates psychotic symptoms in schizophrenic patients, was given to the mice as well. As expected, PPI in the control mice was diminished by ketamine. In the mice born to infected mothers, however, ketamine enhanced PPI, suggesting an abnormality in the glutamate transmitter pathways. Since one theory of schizophrenia posits an imbalance in the dopamine–glutamine systems, the mice born to infected mothers could very well fit in that scenario. Quantitative analysis of these transmitters systems will be required to test this hypothesis raised by the behavioral results.

This influenza model should provide information on how maternal infection may alter fetal brain development (Patterson, in press) and may also prove useful in assessing potential interventions to prevent such alterations.

6. Vaccinology and prevention

6.1. RSV vaccines in adults (Ed Walsh, Rochester General Hospital, Rochester, NY)

At the current time there are several candidate vaccines under development for use in adults. They include purified RSV subunit proteins, subunit peptide fragments and chimeric proteins. All of these incorporate neutralizing epitopes from the dominant F (fusion) and G (attachment) glycoproteins of RSV. In addition, limited work has been published using an intranasally administered, live-attenuated RSV vaccine together with a subunit parenterally administered vaccine. All of these vaccines are in phase I and II clinical studies.

Just as important as selecting a safe and immunogenic vaccine for use in adults is determining which adult populations are most at risk for

serious RSV infection and thus will benefit from vaccination. Respiratory tract infections in various adult populations during the winters of 1999–2000 and 2000–2001 have recently been prospectively evaluated. Groups, which number 200–300 per year, include a nursing home population, adults over 65 years of age living in the community, and adults with underlying high risk conditions (COPD and/or CHF) living in the community. In addition, elderly persons and those with underlying cardiac and pulmonary conditions who were hospitalized with cardiopulmonary symptoms have been evaluated. RSV and influenza virus infections are identified by culture, RT-PCR and serology. RSV infection rates in the prospective cohorts were $\sim 10\%$ annually, and a similar incidence of RSV infection was noted among the hospitalized group. The presence of COPD or CHF was a significant risk factor for hospitalization with RSV, and $\sim 75\%$ of the RSV-infected hospitalized group had one of these underlying conditions, similar to the rate among influenza-infected persons. High-risk elderly have more prolonged periods of being house- and bed-bound, in addition to being more restricted in their activities of daily living. Healthy elderly persons were much less likely to require hospitalization during RSV or influenza infection, although utilization of health care services and illness severity was significant even among this group.

Understanding the source of disease is important in preventing RSV infection. If children are the source of infections in adults, pediatric immunization might reduce attack rates in the elderly. However, we found that among community dwelling adults, 72% with no documented respiratory virus, 70% with RSV, and 87% with influenza A or B were exposed to children. Of patients hospitalized, 52, 54, and 65%, respectively, had been exposed to children recently. The results suggest a somewhat higher risk for influenza than RSV infection relative to exposure to children.

The immune response to infection is also markedly different between younger patients and elderly. Cellular immunity to RSV, as measured by IFN-γ ELISPOTS is lower in the elderly compared to young patients. A 4-fold rise antibody titer as the result of infection was seen against the F antigen in 82% of elderly and 18% of young, against the Ga antigen in 76 and 9%, respectively, and against Gb antigen in 85 and 54% respectively. Neutralizing antibody responses to RSV were also lower among young adults.

Our studies indicate that the annual incidence of RSV infection is approximately 10% and that the impact of disease, although greatest in the high-risk elderly and the immunocompromised. More information is currently needed to understand the impact of RSV on community dwelling adults and to better delineate what components of the immune response to RSV are protective and which are pathogenic. These data also make clear that an RSV vaccine should be primarily targeted to high risk populations, particularly patients with underlying cardiopulmonary disease.

6.2. Safety and immunogenicity of a RSV A vaccine in adults-two phase I studies (Valerie Sales, Aventis Pasteur, Toronto, Canada)

RSV is a common cause of respiratory illness in adults and children. Development of a safe and effective vaccine has been a great challenge. Aventis Pasteur developed an RSV A subunit vaccine with two adjuvant formulations and to conduct two phase I clinical studies. The intramuscularly administered vaccine is composed of co-purified RSV A proteins F, G, and M formulated with either an aluminum phosphate adjuvant (RSV-alum) or with a poly [di(carboxylatophenoxy)phosphazene] adjuvant (RSV-PCPP). The first double-blind phase I study randomized forty 18–45-year-old healthy adults to the RSV-alum vaccine ($n = 30$) or alum-placebo ($n = 10$). The vaccine was generally well tolerated. On day 1, local pain (77 vs. 50%) and tenderness (63 vs. 60%) were reported in both groups of participants; these local events were typically mild and declined in frequency over the week of observation. The difference in pain and tenderness between the vaccine and control groups was only statistically different at 48 and 72 h. Systemic adverse events were uncommon and more frequent in the alum-placebo arm. At 1 month post-vaccination, the vaccine induced a 4-fold or

greater rise in neutralizing antibody titers to RSV A, RSV B and to RSV A and B in 83.3, 76.7 and 73.3% of the vacinees, respectively. A 4-fold or greater rise in anti-F and anti-G antibodies in the treated arm was seen in 73 and 62% of the vacinees, respectively.

In the second phase I study, 40 healthy young adults were randomized to receive either the RSV-alum vaccine ($n = 10$) or the RSV-PCPP vaccine ($n = 30$). Local and systemic adverse events were similar in the two groups, with pain and tenderness at the injection site as the most common adverse events. At 1 month post-vaccination, the RSV-alum resulted in a 4-fold or greater rise in neutralizing antibodies to RSV A in 80%, RSV B in 80%, and RSV A and B in 80% of the participants. The RSV-PCPP resulted in comparable responses in 83, 83, and 77% of the participants, respectively. A 4-fold or greater rise in anti-F antibodies was seen in 80% of RSV-alum and 87% of RSV-PCPP vaccinated individuals while the anti-G response was in 80% of RSV-alum and 77% RSV-PCPP vaccinated individuals.

These two phase I studies document that both formulations are safe and immunogenic, eliciting a greater or equal to 4-fold rise in neutralizing antibodies against RSV A or RSV B in greater than 75% of the participants. The RSV A-based vaccine is able to elicit high levels of neutralizing antibodies against RSV B, such that protection against both RSV A and RSV B subtypes would be expected. The two formulations had comparable safety and immunogenicity profiles. These studies support further clinical development of these RSV subunit vaccines which are promising vaccine candidates for seropositive populations including the elderly, older children, and high-risk patients.

6.3. Update on the cp45 candidate parainfluenza virus 3 vaccine (Theodore Tsai, Wyeth Pharmaceuticals, St. Davids, PA)

Parainfluenza virus 3 (PIV3) is second only to RSV as a leading cause of lower respiratory tract illness in infants (Glezen et al., 1984, 1986). An effective combined vaccine could reduce significantly the burden of acute respiratory illness among infants and young children. A Phase I clinical trial recently was completed to evaluate infectivity, tolerability and immunogenicity of live-attenuated PIV3 and RSV candidate vaccine strains when administered simultaneously in a single intranasal dose. The *cp*45 strain of PIV3 was derived by Belshe and Hissom by serially passaging a human isolate at reduced temperatures, yielding a cold-adapted (ca), temperature sensitive (ts) virus with a reduced replicative capacity in the lower respiratory tract of non-human primates (Belshe and Hisson, 1982; Hall et al., 1993). Multiple mutations in N, C, M, F, HN and L proteins and in the 3′N region contribute to these phenotypic changes from the wild type parent (Skiadoupolos et al., 1999). In several clinical trials, in infants as young as 1 month, the strain was well tolerated and immunogenic and, in a two dose regimen, the first dose provided protection against 'challenge' from the second dose, as reflected in reduced nasal viral shedding (100% shedding after first dose vs. 29% shedding after second dose) (Karron et al., 1995).

The RSV 248/404 strain, derived by Murphy and NIH colleagues, exhibits similar phenotypic ts and attenuation characteristics and also has been shown to be well tolerated and immunogenic in infants as young as 3 months. In 1–2 month old infants, the strain was immunogenic and also induced protection against 'challenge' from a second dose. However, vaccine virus infection produced a brief syndrome of mild nasal congestion that interfered with feeding and sleeping, to a degree that the strain was considered too reactogenic in this age group of young infants (Wright et al., 2000). Nevertheless, the successful experience with the strain in older infants suggested that RSV 248/404 could be combined with PIV3 *cp*45 in a clinical trial in older infants as a proof of principle of combined intranasal vaccination.

In a multicentered, placebo-controlled, double-blind study, 54 doubly seronegative 6–18 month old healthy infants were randomized 1:1:2:1 to receive one of the two monovalent vaccines, the combination vaccine (containing 10^5 pfu of each virus) or placebo as a single 0.5 ml intranasal administration. Viral shedding patterns were followed by infectivity titrations of serial nasal

washes and clinical symptoms were followed with diary cards and clinical examinations. Viral shedding of RSV 248/404 after administration in monovalent or combination formats was similar to the pattern observed in previous trials, with a mean duration of 14 days and mean peak titer of approximately $10^{2.8-3.4}$ pfu/ml between days 8–10. Shedding of PIV3 *cp*45 after its administration alone also conformed with previous experience, with shedding over 14 days, peaking at $10^{3.8}$ pfu/ml on day 7. In the combination group, however, the proportion of vaccinees who shed PIV3 *cp*45, the mean peak titer of shedding, and days of viral shedding all were reduced compared to the monovalent group, although only the last was significantly different. A preliminary analysis of serum immune responses to vaccination shows a similar trend, with an equal proportion of monovalent and combination subjects responding with antibody rises to RSV, while a smaller proportion of combination vaccine recipients had antibody rises to PIV3. Wild type RSV and PIV3 and other viruses circulating in the community infected some subjects, as demonstrated by antibody rises in vaccine and placebo recipients and by viral isolation, even though the trials were confined to weeks when surveillance did not detect epidemic RSV transmission. Respiratory symptoms and fever were common and similar in frequency in all groups and no LRIs or serious adverse events were observed. Contrary to previous experience with the RSV248/404 strain, one third of the recipients of monovalent vaccine developed otitis media; however, the contribution of frequent nasal washes in this trial (5 in the first week) in promoting this adverse event cannot be ruled out. Other clinical studies to evaluate the safety of PIV3 *cp*45 are underway, including a 392 subject study to measure the occurrence of otitis media and other safety events after vaccination; safety among wheeze-prone children; and vaccine strain transmission and stability.

In summary, simultaneous administration of the RSV and PIV3 candidate strains was successful in infecting the majority of recipients, but larger trials are needed to confirm safety and to explore preliminary observations suggesting a modest degree of interference in the replication of PIV3 *cp*45, with the inclusion of the final RSV vaccine strain candidate in the combination. A two dose scheme to assess resistance to the second dose, as a measure of protection and to identify its correlates would be useful.

6.4. Effectiveness and economics of influenza vaccines (Kristin Nichol, The Minneapolis VA Medical Center, Minneapolis, MN)

Influenza is a significant medical problem within the US. Each year, 25–50 million individuals are infected with influenza resulting in 75 million lost work days and 50 million lost school days. The infection also causes 150 000 excess hospitalizations and 20–40 000 excess deaths each year. It is clear, then, that measures to prevent infection should be used if they are effective in reducing influenza associated morbitidy and mortality.

Observational studies spanning many seasons and from different countries have consistently shown that influenza vaccination results in a 20–40% reduction in pneumonia and influenza (P and I) hospitalizations and a 20–60% reduction in mortality among the elderly (Nichol, 1999b). In an analysis of about 1900 elderly with chronic lung disease over 3 seasons, we were able to show that the seasonal variation in hospitalization was not seen among patients who received an annual influenza vaccination (Nichol et al., 1999a). In another study, annual influenza vaccination was effective in reducing the admission rate of P and I by 39%, all acute and chronic respiratory conditions, and congestive heart failure admissions by 27%, in addition to reducing the all-cause mortality by 50%, irrespective of the risk status of the elderly patient. In all, the vaccine resulted in a $73 per person cost savings (Nichol et al., 1998). In another study, 125 000 elderly from 3 large HMO's were followed during the 1997–1998 influenza season to better assess the benefit in high vs. low risk elderly. Event rates for hospitalization or death in high risk elderly persons were about 4–5 times higher than among low risk elderly persons. The number of elderly needed to vaccinate to prevent a single hospitalization or death from this study was < 60 for high risk and < 300

for low risk elderly persons. There is additive benefit of influenza and pneumococcal vaccination in the elderly as well (Nichol, 1999a).

Healthy, younger adults benefit from influenza vaccination, although in a different way than the elderly. Vaccination results in 29% relative and 5% absolute risk reduction of clinical illness and 65% reduction of serologically confirmed influenza infection. The reduction in clinical and serologic infections is greater when there is a good match with the circulating strains of influenza than with poor match (Demicheli et al., 2000). Influenza vaccination is also effective in reducing physician visits for influenza-like illness by 34–44%, reducing antibiotic use by 32–45%, and by reducing sick leave by 25% (Ahmed et al., 2001). Despite all of these benefits, the economics implications of influenza vaccination in this population have been unclear, with some individual studies finding net savings and others finding net costs (Nichol et al., 1995; Bridges et al., 2000). The reasons for these broad differences include wide variations in the observed work loss reductions seen with vaccination. Studies relying on economic modeling can provide additional insights into the economic implications of influenza vaccination among healthy adults. A cost–utility analysis from the 1980's demonstrated that vaccination could be highly cost effective with costs of $260 per year of healthy life gained (YHL) for age 25–44 and $95 per YHL for ages 45–64 for influenza vaccination, expressed in 1999 dollars (Riddiough et al., 1983). A recent cost–benefit analysis suggests that vaccination may be cost saving. This analysis took the societal perspective and used nationally representative estimates to accommodate year-to-year variability of influenza and vaccine effectiveness and to incorporate plausible ranges in other clinical and cost variables. The model, which used Monte Carlo simulation and included sensitivity analyses, incorporated the direct and indirect costs of the vaccine, its administration, and its side effects as well as such averted costs as doctor visits, hospitalizations, work loss avoided, reduced effectivenss avoided, and preserved future earnings. The estimated mean costs incurred per person vaccinated were $16.69 while the mean costs prevented were $30.34, resulting in a net savings of $13.66 ($0–28.99 5th to 95th percentiles) (Nichol, 2001).

In summary, vaccination of the elderly provides substantial health benefits and cost savings, with the highest benefit among high-risk elderly. Vaccination of healthy younger adults reduces illness, provider visits, and work loss. It is also likely to provide economic benefits. As a result, influenza vaccination may be justifiable for all adults, unless prioritization to higher risk groups is mandated by delayed or short supply of vaccine or for other reasons.

6.5. Intranasal influenza vaccine update (Kristin Nichol)

The current live-attenuated, cold-adapted influenza vaccine traces its routes to the master strains developed at the University of Michigan in 1960 (influenza A) and 1966 (influenza B). The annual update of the live-attenuated influenza vaccine (LAIV) is achieved by creating 6:2 master seed viruses from the cold-adapted master strains that undergo re-assortment with the current wild-type strains. The re-assorted vaccine viruses are attenuated, cold-adapted and temperature-sensitive while containing the appropriate hemaglutinnin and NA genes for that season. Potential advantages of the LAIV vaccines include the intranasal route of administration as well as the induction of a broad immune response that is both mucosal as well as systemic. Over the years, many studies have demonstrated its safety and efficacy, and a commercial LAIV (Aviron) is currently undergoing FDA review for approval in children and adults.

The FluMist® (Aviron) LAIV has been administered to over 20 000 children in clinical trials that have demonstrated the safety and efficacy of the vaccine. In the pediatric efficacy trial AV006, (Belshe et al., 1998, 2000) in which 1602 children age 15–71 months were enrolled, children received one or two doses of vaccine during the first year of the trial in a 2:1 randomization (vaccine:placebo). During the second year of the trial 1358 children were revaccinated. The overall efficacy of the vaccine over the 2 years was 92%

against influenza A and 91% against influenza B illness. During year 1 of the trial, vaccine efficacy appeared to be similar for children receiving one or 2 doses of the vaccine (89 vs. 93%). Overall, there was marked difference in the cumulative percent of children who were infected by influenza in the vaccinated children (3%) relative to the unvaccinated children (30%) at 2 years. There was a reduction in febrile illness (94% reduction in influenza culture positive, 23% reduction in all patients), otitis media requiring antibiotics (96% and 30% reductions respectively). There were also fewer influenza-associated lower respiratory tract complications in children who received the vaccine (95% reduction).

The AV006 trial showed that the LAIV was safe and generally well tolerated. However, runny nose or nasal congestion (27 vs. 18%) and fever (6.5 vs. 1.6%) were more common in the vaccine recipients after the 1st dose of vaccine during year. No significant differences were seen after the 2nd dose in year 1 or after revaccination in year 2. A cost–benefit analysis was conducted for the LAIV vaccine in children and resulted in break even vaccine costs of $28 for group-based vaccination programs and $4.93 for individual-based vaccination programs from the societal perspective. The break even cost of either type of program from a third party payer perspective were $10.29 (Luce et al., 2001).

The FluMist® LAIV has also been tested in over 5000 adults, some of whom had underlying immunodeficiency ($n = 57$ with HIV infection) or underlying lung disease ($n = 2215$). In the largest study of working adults, 4,561 adults were randomized 2:1 to receive LAIV or placebo. The study outcomes used clinical definitions of illness. The vaccine was able to reduce the proportion of persons with any febrile illness by 9.7%, severe febrile illness by 17.4%, and febrile upper respiratory illness by 21.9%. There was also a 24.8% reduction in illness days, 28.4% reduction in missed days of work, 40.9% reduction in provider visits, and 45.2% reduction of days of antibiotics prescribed (all with $P < 0.001$). Although more vaccine than placebo recipients had a runny nose or sore throat after immunization, the vaccine was generally well tolerated and most adverse symptoms were mild and short in duration (Nichol et al., 1999b). A smaller experimental volunteer challenge study of protection against influenza A and B viruses induced by LAIV and the trivalent inactivated vaccine found a marked reduction of laboratory-documented influenza illness in recipients of the LAIV compared to placebo. Vaccine efficacy rates were not significantly different for the 2 vaccines 85% (95% CI 28–100%) for LAIV and 71% (95% CI 2–97%) for the inactivated vaccine (Treanor et al., 1999). Finally, as yet unpublished data from a recent cost–benefit analysis for young, healthy adults found a mean break even vaccine cost of $43 (95% CI $22–70) for LAIV use among healthy working adults.

In summary, the FluMist® LAIV is highly effective in preventing influenza in healthy children and young adults. It is generally safe, well-tolerated, and easy to administer. Current studies are being done to look at coadministration with the MMRII® and Varivax® vaccines at 12–15 months. Studies are also planned to look at extending the pediatric community effectivness trial into a fourth year, to assess the safety of this vaccine in children with wheezing illness or asthma, and to look at coadministration with the Prevnar®, Hib, DtaP, and IPV vaccines at 15–18 months.

6.6. Berna virosomal vaccine (Reinhard Glück, Berna, Berne, Switzerland)

Nasally administered influenza vaccination has focused, in the US, on a live-attenuated candidate vaccine. In contrast, a non-replicating virosomal intranasal vaccine has been tested and approved for use in Switzerland. Nasalflu Berna is a trivalent influenza virus vaccine consisting of influenza virosomes which are formulated from inactivated influenza surface glycoproteins, lecithin, and the heat-labile toxin (Escherigen®) from *E. coli* bacteria strains as mucosal adjuvant (HLT). The particles are 150 nm in diameter and are delivered by a nasal spray device in a phosphate buffered saline solution.

Preliminary testing in multiple animal models showed the vaccine to be safe and immunogenic. Mice had a good response to the vaccine with

increased levels of IgA and IgG in the lung BAL and blood. The mice required two doses to achieve an adequate IgA response. In ferrets, the vaccine was able to inhibit fever, inflammatory cell influx, and weight loss following experimental infection. The ferrets also demonstrated a marked reduction in viral titers (<1 log vs. 3–4 log) as compared to unvaccinated animals. Extensive toxicity testing was conducted in several primate and non-primate animals and found no serious toxicity even at doses as high as 40 000 times the usual human dose.

The clinical preparation uses 7.5 mcg of HA antigen per strain, 70 mcg lecithin, and 2 mcg Escherigen®. It is administered twice over a 1 week period, since there was markedly improved immunogenicity with two doses relative to one dose. The two dose vaccine regimen was able to induce a $\geqslant$4-fold rise in IgG antibody titers in recipients to H1N1 (40%), H3N2 (85%), and B (60%). The vaccine had an 85% efficacy in a trial of 250 adults and an 89.7% efficacy in 250 children (Glueck, 2001). No significant adverse events were noted in any of the studies. As a result, the vaccine was licensed for use in Switzerland in 2000.

The vaccine was widely accepted and used during its first season. During the 2000–2001 influenza season, over 100 000 people were given the vaccine. Reports of a 43 cases of Bell's Palsy in recipients of the vaccine occurred and the pharmaceutical company that makes the vaccine suspended sales to investigate the relationship in more detail. Berna sent a survey to 20% of Swiss physicians and found that there were 1200 cases of Bell's Palsy in the country as a whole; this number of cases appeared higher than the usual rate. Preliminary analysis failed to show a clear correlation between time after vaccination to development of Bell's palsy, but further investigation of this potential side effect is ongoing.

6.7. Genetic approaches to influenza vaccine development (Peter Palese, Mt. Sinai School of Medicine, New York, NY)

As the genetic make-up of viruses is better understood, genetically altered influenza viruses have become available as potential vaccine candidates. Recently, the NS1 protein has been identified as a virulence factor for influenza viruses. When the protein is absent, the virus is highly attenuated as the protein is responsible for interferon antagonist activity. When grown in culture systems or in Stat-1 knock-out mice, which are unable to produce interferon, the NS1 deficient influenza virus regains wild-type virulence (Garcia-Sastre et al., 1998). As a result of these findings, several influenza virus constructs were developed with truncated NS1 genes in a PR8 influenza virus. An experiment was conducted in which mice were immunized with viruses containing either wild-type or altered NS1 genes. The mice were then challenged with wild type virus 4 weeks after immunization. Mice that were challenged after immunization with mutant virus survived (Talon et al., 2000). Protection was not seen in mice immunized with low doses of a virus lacking the NS1 gene (3.3×10^4 pfu vs. 1×10^6). Currently studies are being planned to assess the efficacy of NS1 deficient A/Texas/91 virus-based vaccines in humans.

Because the NS1 gene appears to confer virulence, the NS1 gene of the 1918 influenza virus was analyzed and compared to that of the PR8 virus used in the mouse experiments. A virus containing the 1918 NS gene was created and grown in tissue culture. This strain appeared not to be virulent and did not induce death in infected BALBc mice, while the PR8 virus caused universal fatality at 10^4 PFU inoculations. This finding suggests that the NS1 protein of the 1918 virus may possess a species-specific activity because it is unable to counteract the interferon response in a mouse (Basler et al., 2001). Whether the 1918 NS1 protein confers a high virulence phenotype in humans remains to be determined.

Another recent advance has been the use of Newcastle disease virus (NDV) as a vaccine vector. An influenza virus HA gene has been spliced into the genome of NDV. The recombinant virus is stable and able to effectively produce influenza antigens without causing disease in mice. A study in which mice were given $3x10^7$ pfu of rNDV/B1-HA at day 0 and 21 and then challenged with 100 LD50 of A/WSN/33 on day 35 was done. The

vaccine was able to produce high level HI titers to the A/WSN/33 virus in the vaccinated mice, whether vaccine was given IV or IP. The vaccine attenuated weight loss and allowed 5/5 mice to survive when challenged, while none of the unvaccinated mice survived (Nakaya et al., 2001).

These studies suggest that future influenza vaccines may be derived from genetically altered influenza viruses or from other viruses including NDV that have been genetically altered to produce protective influenza antigens in the host. These exciting developments warrant further study.

6.8. Boosting H5N3 immunity in a primed population (IainStephenson, Leicester Royal Infirmary, Leicester, UK)

Over the past several years, there has been heightened concern about the emergence of H5N1 influenza strains as the cause of the next influenza pandemic. Prevention of this virulent agent could best be accomplished by vaccination. A recent study was conducted to establish the utility of a novel MF59 adjuvanted vaccine. The vaccine is prepared as a MF59 oil-in-water emulsion solublized with squalene and polysorbate 80 with H5N3 surface antigen soluablized with sorbitan trioleate and citrate buffered sterile water. An observer blinded, randomized, dose-ranging study was conducted in 60 healthy young adults (Nicholson et al., 2001). Patients received 2 intramuscular doses, 3 weeks apart, of either 7.5, 15, or 30 μg HA content of the MF59 adjuvanted or plain vaccine. Microneutralisation (MN) was tested against the H5N3 A/Duck/Singapore/97, hemagglutination inhibition was tested against A/Beijing/262/97 (H1N1), A/Sydney/5/97 (H3N2) and A/Duck/Singapore/97 (H5N3), and single radial hemolysis (SRH) was tested against the A/Duck/Singapore/97 (H5N3) and A/Hong Kong/489/97 (H5N1) viruses. These studies demonstrated that HI is insensitive for the detection of H5 antibody compared to MN and SRH. MF59 adjuvanted vaccine was well tolerated and had a greater antibody response at day 21 and 42 than unadjuvanted vaccine. A follow-up study was conducted to determine the durability of response and residual immunity at 12 months and to assess the effect of a single additional H5N3 vaccination on immune responses in the primed group. The follow-up study was unblinded and the patients received the same dosage and vaccine as they received in the initial study. MF59 adjuvanted vaccine was again well tolerated. Fifteen months after receiving the first two vaccines, there was no detectable immunity by HI or MN method, although there was a small detectable residual antibody level by SRH (greatest in the 30 μg group) in the MF59 adjuvant vaccine group only. Twenty-one days after a booster dose of vaccine, there was minimal response by HI and modest by SRH in the non-adjuvanted arm. The response was far more robust in the MF59 adjuvanted arm, with a maximum geometric mean titer of 1:32 by HI and 130 mm^2 by SRH to the H5N3. The SRH for H5N1 was nearly 90 mm^2. The degree of response, irrespective of the method used, was higher with the booster dose at month 15 than it was after the second dose in the initial series. Finally, the number of patients who seroconverted by HI was 9/15 and 0/11 21 days after booster vaccination in the MF59 and plain vaccine groups respectively; seroconversion by SRH against H5N3 and H5N1 was 15/15 and 8/11, respectively. These two studies were able to demonstrate that the MF59 adjuvanted vaccine resulted in a greater response than the unadjuvanted vaccine and that re-vaccination at 15 months was very successful in boosting the immune response, particularly in the MF59 adjuvanted group. This data also suggests that HI responses generally underestimate the antibody response as compared to MN and SRH.

6.9. A new influenza vaccine for nasal administration using chitosan as an absorption enhancer (Roy Jennings, Sheffield University, Sheffield, UK)

Current influenza vaccines are formulations of inactivated preparations administered parenterally, containing either the surface HA and NA proteins of the virus or whole virus particles. They are suboptimal vaccines, in that they induce immunity, at best, in only 60–90% of vaccinees, and

individuals at the extremes of age respond particularly poorly. Respiratory mucosal immune responses contribute significantly to natural immunity, and a strategy for improving the current influenza vaccines may be administration of influenza vaccines via a mucosal route. In animal studies, influenza vaccines incorporating a variety of adjuvants and delivered by an intranasal route have reported good local and systemic antibody responses and protection against subsequent challenge infection.

A new influenza vaccine that incorporates chitosan, a de-acylated form of chitin obtained from shrimp and crab shells with the current, commercially-available subunit influenza virus vaccine has recently been developed (Solvay, Weesp, Netherlands). An initial study administered this chitosan-influenza vaccine formulation to healthy adult volunteers aged from 18 to 60 years. Studies in both animals and humans indicate that the mucoadhesive properties of chitosan can enhance the bioavailability of proteins following intranasal administration. Chitosan has been shown to be safe in humans, with no nasal toxicity and no adverse effects on mucociliary clearance. The study, carried out in 1999, used the commercial subunit influenza vaccine which contained H3N2 (A/Sydney/97), H1N1 (A/Beijing/95) and influenza B (B/Yamanashi/98) antigens, and this was mixed, at two concentration levels, with 10 mg/ml of chitosan. Both full-strength (15 μg) and half strength (7.5 μg) amounts of each of the viral antigens was used in the experimental vaccine. In addition the commercial influenza vaccine, containing 15 μg of each of the viral antigens, without chitosan, was employed as a 'gold' standard.

The study recruited 61 volunteers, 23 of whom received the full-strength chitosan-influenza vaccine, and 21 the half-strength chitosan-influenza vaccine preparation by intranasal spray on 2 separate occasions 28 days apart; 17 volunteers were given a single dose of the commercial vaccine by the intramuscular route. Blood samples were collected prior to each vaccination, and at regular intervals subsequently.

The results showed the HI antibody seroconversion rates against the A/Sydney moiety of the vaccine to be similar following both intranasal delivery of the full strength chitosan-influenza vaccine and the intramuscularly-delivered, unadjuvanted commercial vaccine. However this was not the case for the influenza H1N1 and B moieties of the vaccine where the seroconversion rates were greater following intramuscular vaccine delivery. Nevertheless, with respect to both the H3N2 and H1N1 influenza type A virus HA antigens in the vaccine, intranasal delivery of 2 doses of the chitosan-influenza preparation at full strength elicited seroconversions in more than 40% of the volunteers, thereby meeting Committee for Proprietary Medicinal Products (influenza vaccines) (CPMP) requirements. Furthermore, for the A/Beijing vaccine component, the half-strength preparation of the chitosan-influenza vaccine also met this requirement.

The requirement of the CPMP in respect of protection rates, (serum HI antibody titres equal or greater than 1 in 40), for experimental influenza vaccines in healthy adults, is that such vaccines should achieve this in 70% of the vaccinees. In the present study, this level was achieved, following 2 vaccine doses, with respect to the H3N2 (A/Sydney) and influenza B chitosan-influenza vaccine components, at both full- and half-strength dosage levels, and also at the half-strength dosage level for the H1N1 (A/Beijing) chitosan-influenza vaccine component. All three vaccine components of the intramuscularly-admimistered, standard commercial vaccine induced protection rates above the CPMP requirement levels.

The nasally-administered, chitosan-influenza vaccine used in the present study was well tolerated by the volunteers. For the full strength preparation, 79 adverse events were reported, of which 94% were mild, primarily rhinitis of short duration, and none were severe. With respect to the half strength chitosan-influenza vaccine preparation, 72 adverse events were reported, of which 89% were mild. There were two severe adverse events in this group, both unconnected with the vaccination program. In summary, the 1999 the influenza vaccine study showed that nasal administration of this preparation is safe, well-tolerated and can elicit serum HI antibody responses that, at least for some components of the vaccine, reach

levels of seroconversion and protection that comply with CPMP guidelines for experimental influenza vaccines in healthy adult volunteers.

7. Clinical features and diagnosis

7.1. Diagnosis in the elderly: is it RSV or is it influenza? (Ed Walsh)

The overlap in clinical presentation of RSV and influenza is significant among elderly persons and those with underlying COPD or CHF. This is true for both hospitalized persons and outpatients. However, influenza infected persons tended to have higher temperatures, while RSV infected persons have higher rates of wheezing and nasal congestion. Influenza is also more often associated with chest pain, rhonchi and rales, an infiltrate on chest X-ray, and a short duration of illness. RSV infection is more often associated with sputum production, dyspnea, and a longer duration of illness. Diagnosis of RSV in adults depends upon laboratory tests, such as culture, RT-PCR, or serology. Among 1495 adults evaluated, 166 RSV infections were identified; RSV culture was positive in 4.1% of the 1495 subjects, PCR in 9.0% and serology in 12.4%. Overall, we found the sensitivity of PCR to be 77% among outpatients and 69% among hospitalized persons, with a specificity of 99% for both. In contrast, culture was only 39% sensitive and rapid antigen detection 9–23% sensitive.

7.2. Predictive value of clinical presentation (Arnold Monto, University of Michigan, Ann Arbor, MI)

While there is considerable overlap in symptoms associated with the various respiratory viruses, differences do exist. Advantage can be taken of these differences in developing clinical predictors of the specific viral etiology of respiratory infections. These predictors can be used in a number of ways. Currently, a principal one is to determine situations in which influenza antivirals should be used for treatment. At least two studies have shown that cough and fever are the most important predictors of type A and B influenza (Monto et al., 2000a; Monto and Ohmit, 1996). Of note, the degree of temperature elevation appears to increase the likelihood of influenza in addition to correlating with the time to relief of symptoms (Monto et al., 2000b). However, when the data again came from physician-based surveillance of all respiratory illness over periods of high and low influenza virus activity, the positive predictive value (PPV) of cough and fever was at most 40% (Carrat et al., 1999). In contrast, demonstration that influenza transmission was occurring in the community was a prerequisite for enrollment in studies of antiviral treatment of influenza. Thus, influenza infection frequency was higher than when the entire winter season was followed. When data from the zanamivir studies were examined, it was found that the PPV of cough and fever for influenza positivity was 79%. The higher the fever, the better the predictive value. Of note, these are also the patients who benefit most from antiviral treatment. In a Canadian study similarly conducted during a period of influenza virus transmission, the PPV of cough with fever greater than 38 °C was 87%. In part, the high PPV is a result of high prevalence of influenza infection (Boivin et al., 2000).

A particular problem is the differentiation of influenza from other respiratory viruses in children during the influenza season. A recent study found that cough (OR 1.7), fatigue (OR 1.6), headache (OR 2.2), myalgias (OR 2.7), and chills (OR 2.2) were strongly related to influenza infection. Expiratory wheeze was a good predictor of other infections (OR 0.3). Although fever above 37.8 °C had an OR of 4.3 predictive of influenza infection, it was not significant. (95% CI 0.6–32.3, $P = 0.16$) (Manuguerra et al., 2001).

Data on other respiratory viral infections is more limited. From the studies of pleconaril, two symptoms, congestion and rhinorrhea, appear to be more common in rhinoviral colds. Unfortunately, the difference between rhinoviral and non-rhinoviral colds for congestion (54 vs. 41%) and rhinorrhea (72 vs. 55%) is small.

Some have suggested that rapid tests for influenza might be useful at times when influenza viruses are circulating sporadically. However,

even assuming good sensitivity of these tests (70%), the PPV similarly falls in periods of low virus transmission, indicating that many true positives will be missed. Moreover, laboratory studies using frozen specimens have shown that the sensitivity may be much lower (Table 1). Of interest, these data suggest a markedly lower sensitivity of the Directogen A and B in detecting influenza A viruses compared to the Directogen A test, which is the most sensitive in this evaluation. Some outbreak studies have found problems with specificity; among 14 positive tests (2 by Quickvue and 12 by Flu OIA) from 3 different nursing homes, all samples were negative by cell culture and PCR. Such false positive tests have resulted in outbreaks incorrectly being identified as caused by influenza.

In summary, the symptoms of cough and fever consistently predict influenza virus infection when the influenza prevalence is high, and the predictive value is improved with increasing fever. Influenza infection can be predicted in adults with as much ease as in children under the age of 5. Rapid tests will be least helpful when influenza virus prevalence is low, and care must be taken with these rapid tests as their sensitivity and specificity is not yet optimal for all situations. Finally, prediction of rhinovirus positivity may be possible using similar clinical predictive models.

7.3. Management and analysis of large genetic sequence data sets (Catherine Macken, Los Alamos National Laboratory, Los Alamos, NM)

Over the past decade, technologic advances have allowed the sequencing of the genomes of several organisms. These sequences tend to be large and difficult to share. Recently, the Influenza Sequence Database (ISD) (http://www.flu.lanl.gov/) was established at Los Alamos National Laboratory in the Theoretical Biology and Biophysics Group (T-10) to serve as a site in which influenza genomic sequences are stored, analyzed, and shared. The Influenza Sequence Database is a curated database of nucleotide and amino acid sequences. It is intended to provide the research community with easy sequence deposit and retrieval capabilities, together with tools tailored, in particular, to the analysis of HA and NA sequences. Care has been taken to remove redundant sequences and to fill in missing field data. The project began in the spring of 1998; the database now holds all influenza A, B, and C sequences that have been published in GenBank, as well as a growing number of unpublished sequences. Researchers interested in depositing any of their unpublished sequences directly into the ISD can do so via a password-protected web site or via e-mail directed to Catherine Macken, (cmacken@lanl.gov).

In order to provide search and 'Blast' services that do not pull up redundant entries, all new sequences are reviewed as they are received to ensure that they are of adequate quality and completeness by comparing them to previous sequences. If two or more GenBank nucleotide records correspond to the same influenza strain and contain identical sequences, we classify all except one as a 'duplicate.' Currently, we take that protein sequence cross-referenced by the PID in a GenBank nucleotide sequence to be the primary protein sequence, if it exists. If the GenBank nucleotide sequence was not translated into a protein record, we take a protein sequence from another database: this latter event rarely occurs. In the case of a few strains, duplicates result from

Table 1
Sensitivity of antigen detection tests from frozen specimens using cell culture as the gold standard (Monto et al., 2001)

Influenza type	Flu OLA sensitivity (%)	Quickvue sensitivity (%)	Directogen A and B sensitivity (%)	Directogen A sensitivity
A H3N2	22	22	50	89%
A H1N1	0	0	0	40%
B	0	0	66	NA

structural studies, where secondary or tertiary structural information changes while the sequence does not. We classify duplicate sequences from structural studies as 'structural duplicates' of the primary protein sequence that they match. Finally, another source of duplicate record is the resequencing of a gene that was originally only partially sequenced, with more extensive coverage of the gene by the later sequencing effort. In this situation, we classify all except the longest sequence as a 'duplicate,' provided that the sequences are identical in their region of overlap.

The data is stored in a relational database that allows correlation of epidemiologic, genetic, and virological information. Users can perform basic or more advanced searches, as well as two specialized functions called Region and Prosite search. Region search is an advanced search with the additional feature of allowing the user to prune returned sequences to a region of interest. Region search is based on prealigned sequences, and hence can be used to download alignments. Prosite search is another advanced search, applicable to protein sequences only, with the additional feature of identifying 'prosite' patterns. Prosite search can be used to color-code glycosylation sites, CTL epitopes, or other patterns of interest onto pre-aligned protein sequences. The site also includes analytical tools that allow the user to select and highlight residues on a dynamic visualization of the crystal structure of H3 HA, using RASMOL. Another tool translates accession numbers on a user-supplied phylogenetic tree into full or abbreviated strain names. More tools are in the development stage.

All of this data is available in multiple formats and is downloadable. A recent innovation is the capacity to house private compartments on the site. With these compartments you can use the public data in the site with your data but your data is only visible to you. For example, to serve the needs of the Neuraminidase Inhibitor Susceptibility Network (NISN), a private compartment was created for their use. The public can view the database, but only members of NISN can view their compartment of the database.

This database is an expensive undertaking and requires a number of highly trained and highly skilled individuals to maintain. It is constantly being improved upon by input of its users.

7.4. Adenoviral respiratory infections in young adults in US military training (Margaret Ryan, Naval Health Research Center, San Diego, CA)

Outbreaks of respiratory illness due to adenoviruses have been described in military recruits since the 1950s. This increased risk is in part due to stresses of training, environmental factors, and sudden mixing of susceptible young adults in close contact settings. Adenovirus was associated with a 50–80% attack rate. Adenovirus caused 10% of all recruits to be hospitalized in 1958 and 72% of acute respiratory illnesses in military personnel during winter months were attributable to adenovirus. Oral, live attenuated vaccines against adenovirus serotypes 4 and 7 were developed and by 1971 all new military recruits were required to receive the vaccine. The vaccine was solely produced by Wyeth Lederle Vaccines which discontinued production of the vaccine in 1995. Remaining supplies were used, in a more limited fashion from 1996–1999, but no usable vaccine currently remains. To address the loss of the vaccine, the US Department of Defense awarded a contract to Barr Pharmaceuticals to begin making the vaccine. Unfortunately, this vaccine will not be widely available until 2006.

The Department of Defense's Center for Deployment Health Research (http://www.nhrc.navy.mil) was given the task of assessing the current impact of adenovirus in the military in an attempt to provide information to help guide vaccine development and to assess various control methods (Ryan et al., 2000). The Center for Deployment Health Research's surveillance at 8 US military basic training sites includes collection of population demographics and clinical presentation data. Young adults with febrile respiratory illness provide throat swab specimens that are tested for the presence of viruses in cell cultures. Since the discontinuation of routine vaccination against adenovirus, there has been a trend toward more cases of adenoviral respiratory infections (averaging 3 cases/1000 recruits/week in 1998; 3/1000/week in 1999 (the last year of avail-

able vaccine); 6/1000/week in 2000; and 6/1000/week in 2001). Additionally, there have been epidemic adenoviral outbreaks, defined as greater than 1.5 cases/100 recruits/week, in military basic training camps every year since the cessation, which is in stark contrast to more rare epidemics with vaccination (Gray et al., 2000). More than 22 800 adenoviral illness cases occurred in these populations during 2000, (Ryan et al., in press) including 2 fatal cases (Ryan et al., 2001).

Most cases are still caused by adenovirus type 4, followed in frequency by type 7, 3, and 21. Unvaccinated recruits are 13 times more likely to have a positive adenovirus culture (OR = 13.2: 95% CI 10.7–16.2) and 28 times more likely to be positive for type 4 or 7 adenovirus (OR = 28.1; 95% CI 20.2–39.2) than vaccinated personnel. Most cases of adenovirus infection present with a fever greater than 102 °F and are complicated on average by 3 days of reduced activity and 10 days of respiratory disease. Patients with infections caused by type 7 adenovirus typically present with nasal congestion (96%), sore throat (71%), cough (68%), and gastrointestinal disturbances (46%). Using multivariant analysis, the Center has concluded that lack of vaccination represents the greatest risk for adenoviral infection, with an odds ratio greater than 20 in most models. Additional risk factors include autumn recruitment, home state of Kansas and New Mexico, and smoking status, with non-smokers having a great risk of type 3 infections. Gender, age, race, past respiratory diseases were not associated with adenoviral infections. Cost-effectiveness analyses have found the vaccine to be beneficial in maintaining the military's readiness for battle (Hyer et al., 2001).

8. Antivirals and other management strategies

8.1. New antivirals in an era of healthcare cost containment (Thomas Szucs, Hirslanden Holding, Zurich, Switzerland)

Over the past several decades, healthcare costs have increased substantially as a result of many factors including new treatments for previously untreatable conditions, new evidence to supporting broader application of existing treatments, an overall increase in the cost of treatments with greater development in areas such as biologics that are more expensive, increased intensity of treatments, quality improvement measures, and increased demand from a better educated public. These enormous growths in cost have led to renewed interest in cost containment. Increasing interest in cost containment is derived from an aging population who, as a result of anti-egalitarian trends, are paying higher co-payments for their healthcare. In addition, we observe an increasing gap between what medicines can do and what is affordable. Hand-in-hand with the increased interest has come a wider use of evidence-based medicine and technology assessment to better assess which costly interventions produce the greatest benefit. Cost containment currently focuses on price reduction, through increased competition and price controls; on demand reduction, through reimbursement limits and managed care; and on budgeting, through global healthcare budgets and, in some countries, certificates of need for drug approval.

Pharmaceuticals overall have a beneficial effect on life expectancy and total health care costs. It is also clear that there is a specific benefit from the use of antivirals, especially the NA inhibitors. The antivirals directly reduce costs by reducing the number of follow-up consultations and antibiotics used and reduce the incidence of complications and their resultant hospitalizations. These direct costs are estimated to average $3.7 billion in Europe and $1.9 billion in the US annually. Indirectly, anti-influenza treatment can improve productivity which contributes to their cost-effectiveness. Indirect costs of influenza amount to $18 billion in Europe and $9.5 billion in the US annually. The incremental cost-effectiveness of zanamivir per symptoms-free gained day by several estimates ranges from $7.25–22.58 based on December 28, 2001 conversion data (Griffin et al., 2001; Mauskopf et al., 2000). Unfortunately, there are no cost-effectiveness analyses that have head-to-head comparability of different therapeutic options, and none have been done with reference to quality adjusted life years

(QALYs). As a result, the UK National Institute for Clinical Excellence (NICE) initially decided not to recommend the widespread use of zanamivir in 1999. It changed its stance in 2000, recommending zanamivir for use in at-risk adults when influenza was circulating in the community and in patients who present within 36 h of onset of illness. The potential impact of this decision, if used for all 97 000–487 000 at-risk individuals, would have yielded a cost of between £2.3–11.7 million. This reversal of positions was brought about by two cost-effectiveness studies which were referenced to QALYs. In the first, conducted by NICE, estimates of £38 000/QALY for all adults when influenza is circulating and £9300–31 500/QALY for at-risk adults when influenza is circulating were based on the assessment of clinical effectiveness estimates of the drug, costs of additional physician visits, and the costs of hospitalizations (NICE Technology Appraisal No. 15). Additional evidence from the NAI3008 study in at-risk patients, that assumed a 6% reduction of mortality, from a baseline of 28/100 000 influenza infected individuals, was added to the first model and resulted in a £21 000/QALY estimate.

Outside of England's NICE, there are changes taking place that will have a profound effect on drug approval relative to their cost-effectiveness. Many countries throughout Europe require two steps in drug approval: a registration of the drug by the Ministry of Health and an agreement on price to allow the new drug to be added to the nation's formulary. This pricing is based on mandated pharmacoeconomic analysis for which nearly every country has an agency that has provided economic guidelines (Celibera CIPE n 109, 1997). Although pharmacoeconomic analyses will likely result in cost savings overall, they are having difficulty in their implementation because harmonization is moving forward in registration of new drugs without similar harmonization in cost-setting practices.

8.2. Neuraminidase inhibitor susceptibility network (Maria Zambon)

Industry, academia, and governments were concerned with the development of antiviral resistance and subsequent impact on influenza disease following the widespread availability and use of neuraminidase inhibitors (NIs). At the point of licensing such drugs it is not known what the impact, if any, that resistance to NI drugs will have on treatment of infection or evolution of the virus, particularly with regard to antigenicity. As a result, the establishment of a resistance monitoring group was a component of the licensing approval of both of the clinically available NI drugs. The Neuraminidase Inhibitor Susceptibility Network (NISN) was established to fulfil this goal (Zambon and Hayden, 2001a). The NISN, which is currently funded by Roche Pharmaceuticals and GlaxoSmithKline, is composed of a core voting membership composed of the WHO collaborating centers (Alan Hay, Alan Hampson, Alexander Klimov, and Masato Toshiro) and members of the academic and public health community (Jenny McKimm-Breschkin, Robert Webster, Arnold Monto, Michelle Aymard, Catherine Macken, Frederick Hayden, and Maria Zambon) as well as observers from the sponsoring pharmaceutical companies and the World Health Organization. Currently the NISN has established a link between the WHO and the two sponsoring companies, validated assays for phenotypic resistance by comparing resistance assay performance against panels of viruses and in mixing experiments with wild type and known resistant mutants. It has also collected and analysed the NA 50% inhibitory concentration (IC50) of influenza isolates collected worldwide prior to the introduction of NI drugs, and selected isolates which are outliers on phenotypic screening for sequencing, to an attempt to correlate phenotypic and genotypic data.

Resistance to the currently available NIs, zanamivir and oseltamivir, occurs as the result of mutations in the NA gene with resultant decreased binding affinity for the NI or of mutations in the HA gene with resultant decreased affinity for sialic acid. Resistant variants have been recovered but with a very low incidence (0.4–4% for oseltamivir, 1 case for zanamivir) and typically late in the course of illness from patients with prolonged shedding. NI-resistant virus due to NA mutations appears less efficient at infecting

new cells, does not appear to be transmitted by humans, and is not associated, to date, with rebound viral replication (Welliver et al., 2001).

Methods for assessing phenotypic resistance have been developed including NA inhibition assays with or without genotyping for NA mutants. Such studies have identified 5 distinct NA mutations associated with resistance to one or both NIs (E119G, R292K, R152K, E119V, H274Y). Mixing experiments comparing fluorometric and chemiluminescent NI methods indicate that the shape of the resistance curve is more dependent on the mutant than on the method used, and that similar IC50 values are obtained between different methods. To date, 1054 samples from 1995–1998 collected before introduction of the NIs and have been studied. Using these samples, the NISN has shown that the IC50 derived from fluorometic analysis is comparable to chemiluminescent methods. Chemiluminescence has been selected as the primary method for screening samples for resistance as it allows the highest throughput with the lowest variability. Using this method, all known resistant variants have an IC50 outside 95% of all of the samples, so a 5% outlier rule was accepted to limit which viruses to analyse further for resistance genotype.

The precise orientation and the immediate surrounding residues of conserved NA site may differ between subtypes and that drug binding may not be identical across all subtypes. From the preliminary data, it appears that different drugs may produce different resistance patterns and that resistance strategies may not be identical across all subtypes of virus. From the analysed isolates, it appears that there is wide variation in IC50s in natural isolates but that there is no significant variation by year or geography. In the future it hoped to conduct sequence analysis of NA and HA of outlier viruses, to analyse the isolates following the introduction of drug use, to delineate the relationship between genotypic and phenotypic resistance, to conduct more detailed analysis of pre- and post-treatment isolates, and to effectively disseminate this data to the community.

8.3. FLUNET®: a novel approach for influenza management (Simon Tucker, Biota Holdings Limited, Melbourne, Australia)

The FLUNET technology was established to discover new neuraminidase inhibitors with a long residence time in the respiratory tract and high potency with the ultimate goal of producing a novel drug that could be dosed once daily for treatment and once weekly for prophylaxis. Cooperative work between Biota Holdings Limited and GlaxoSmithKline lead to the design of several multimeric zanamivir molecules linked through the 7′ hydroxy group. Initial testing of the dimers, trimers, and tetramers was conducted to screen for potential drugs for further development. Screening for in vitro activity against six influenza A and four influenza B viruses revealed that some dimers were extremely potent with a median EC_{50} approaching 0.01 μg/ml in cell culture. Next the number of linker atoms between the two zanamivir molecules were varied and activity assessed against A/Sydney/5/97 and B/Harbin/7/95 viruses. Preliminary experiments showed that the greatest activity was found with linkers of 13–18 atoms with substantially lower activity for shorter linkers and evidence of reduced activity for longer linkers. For example, a dimer with a 14 carbon alkyl linker had an EC_{50} of 0.14 ng/ml for A/Sydney/97 and 0.32 ng/mL for B/Harbin/95 in cytopathic effect assays.

Rat lung retention studies were done by delivering 0.4 mg/kg of the study drug or zanamivir via intra-tracheal instillation. Levels of the dimer within the lung remained at approximately 10 000 ng/g tissue by day 7, significantly in excess of its EC_{50} (median $EC_{50} \sim 0.2$ ng/ml), while zanamivir had declined substantially to a level approaching its EC_{50} of approximately 20 ng/ml. Prophylactic studies were conducted in which mice were dosed once with the dimer or zanamivir and were then infected with influenza 7 days after the administration of the dose. Protection of zanamivir dosed mice was only evident in high dosage groups receiving 1–5 mg/kg, whereas sufficient dimer remained after 1 week to reduce viral titres by over 90% after doses as low as 0.025 mg/kg. Finally, a treatment experiment used zanamivir twice a day

Table 2
Outcomes of therapeutic arms of Ontario Nursing Home Study

Outcome	No treatment (%)	Oseltamivir >48 h (%)	Amantadine <48 h (%)	Oseltamivir <48 h (%)	Prophylactic oseltamivir (%)
Antibiotics used	65	70	29	20	14
Complications	48	35	25	6	0
Hospitalizations	22	17	15	0	0
Deaths	22	4	14	2	0

Table goes with Risebrough talk.

or the dimer once only at 0.1, 1, and 10 mg/kg. At all doses, a single administration of the dimer was found to yield equivalent reduction of viral titres compared to untreated mice as twice daily zanamivir.

Other studies suggested an increased aggregation of virus treated with the dimer relative to monomeric zanamivir, providing a possible explanation for their increased potency. Cross-resistance occurs between the dimers and monomeric zanamivir, which indicates that both act on NA. Currently, Biota is doing lead selection studies to determine which agent would be best to move forward in development.

8.4. Oseltamivir treatment in elderly long-term care residents (Nancy Risebrough, Mount Sinai Hospital, Toronto, Canada)

Therapy with neuraminidase inhibitors is effective in reducing symptom duration and severity in healthy adults infected with influenza, but therapeutic efficacy in residents of long-term care facilities has yet to be fully described. Data was collected as part of a compassionate use program for the management of influenza outbreaks in residential long term care facilities (LTCF) for the elderly in Ontario, Canada during the 1999–2000 influenza season. Over 90% of the residents and staff of the facilities had received influenza vaccination in the fall of 1999. Oseltamivir was offered to residents of 10 facilities during 11 outbreaks of influenza, all due to A/Sydney/5/97 (H3N2). In five outbreaks, transmission of influenza occurred despite amantadine prophylaxis, and oseltamivir was offered to residents for treatment and prophylaxis. In the remaining six, residents were offered their choice of amantadine or oseltamivir when the outbreak was recognized. Data were collected daily or every other day on the status of the outbreak. A retrospective chart review was done to identify antibiotic use, influenza complications, hospitalizations, and deaths. Patients who developed influenza while receiving amantadine prophylaxis were offered oseltamivir 75 mg twice daily for 5 days. Ill residents who had not taken amantadine previously, were offered either amantadine (100 mg QD $\times$ 5 days, dose adjusted for creatinine clearance) or oseltamivir as above. All patients were comparable with regard to age, severity of illness, baseline ADL capacity, and vaccination status. There were 23 patients with influenza-like illness (few patients had culture or PCR documented influenza) who received no therapy, 60 who received amantadine more than 48 h after symptom onset, 23 who received oseltamivir more than 48 h after the onset of symptoms, 50 who received oseltamivir within 48 h, and 14 who had symptom onset while receiving oseltamivir prophylaxis and who continued on oseltamivir therapy. Patients who started oseltamivir less than 48 h after the onset of symptoms or were on oseltamivir prophylaxis had significantly few complications, hospitalizations, and deaths relative to those who received no therapy, amantadine or delayed oseltamivir. Fewer antibiotics were prescribed for those residents who received early, specific influenza therapy (Table 2). Oseltamivir was well tolerated; overall 3 of 87 residents had adverse events (one each with confusion, dizziness, and headache). Treatment of influenza with antivirals during outbreaks in LTCFs is associated with a reduced rate of antibiotic use. Early treatment with oseltamivir

appears to be associated with a reduced rate of serious complications when compared to no treatment or amantadine.

In Canada, amantadine post-exposure prophylaxis (PEP) is the standard of care for ILI prevention in most LTCF. The cost-effectiveness of oseltamivir PEP is unknown. A cost-effectiveness model was developed to examine the cost and outcomes of a management strategy of oseltamivir PEP compared with amantadine PEP and no prophylaxis from a Canadian government perspective in a vaccinated elderly LTCF population, over 30 days. Prophylaxis was initiated following a confirmed influenza A outbreak in the LTCF. Events modeled were drug related adverse events, emergence of ILI while on prophylaxis, use of antibiotics, ILI complications, all cause mortality and resistance to amantadine. We estimated that 40% (31–64%) of LTCF to would have an influenza A outbreak and in those facilities, 17% (2–43%) of patients would develop ILI without prophylaxis. Without prophylaxis, 20% (17–48%) of patients with ILI develop a serious complication and 11% (2–22%) die. Probabilities were determined from randomized controlled trials and observational studies. Based on the available literature, in LTCFs without identified amantadine resistance, amantadine PEP reduced the risk of developing ILI by 60% and oseltamivir PEP by 63%. Costs for drugs, labs, physician visits, and acute hospital days were included based on Canadian government sources and published literature. We assumed all patients receiving amantadine had dose adjustment according to creatinine clearance at a cost of $16.52 (2002 CDN) per patient for laboratory studies, but the cost of pharmacist and nursing time for dose calculation was not included. Results showed that compared to no prophylaxis, oseltamivir saved $3357 per 100 patients and prevented 4.2 ILI cases per 100 patients. Compared to amantadine, oseltamivir saved $1348 per 100 patients and prevented 1.4 ILI cases per 100 patients. The cost of amantadine dose adjustment was a significant cost in the amantadine PEP strategy. Compared to no prophylaxis in this analysis, PEP strategies save overall health care costs despite additional drug acquisition cost. Based on these results, prophylaxis with oseltamivir is a cost-effective influenza prevention strategy in the LTCF environment.

8.5. Treatment of influenza in children-an otolaryngologist's perspective (Birgit Winther, University of Virginia, Charlottesville, VA)

Influenza enters the host by depositing in the eyes or nose, typically by the hands, or by inhalation. The anatomy of the upper airway is optimized to enhance influenza virus deposition in the nasopharynx (Proetz, 1953). After deposition, it readily infects the adenoid epithelium (Winther et al., 1990) but may also spread to the sinuses, eustachian tube, and the middle ear. Recent studies have shown that nose blowing, but not sneezing or coughing, is capable of raising intranasal pressures as high as 70 mmHg. These same studies have used iodinated contrast to demonstrate the passage of material from the nasopharynx to the sinuses with nose blowing (Gwaltney et al., 2000a). Currently, very little information exists on involvement of sinuses during influenza infection in children. In children with common cold symptoms of less than 72 h, the majority (90%) had evidence of sinusitis by CT scan days 1–5 and 41% by days 21–28 (Schwartz et al., 2001). Although difficult to diagnose clinically it is therefore likely that the sinuses are involved during influenza infections in children and may lead to viral, bacterial, or mixed viral–bacterial sinusitis.

Attention has recently been placed on the role of influenza in middle ear infections. Eustachian tube dysfunction and altered middle ear pressures, detectable by tympanogram, occur in the influenza infected patient. Prophylactic intranasal zanamivir in adults infected experimentally with influenza is effective in preventing an abnormal tympanogram (15 vs. 61% of placebo treated patients) (Walker et al., 1997). Influenza appears to be a risk factor for AOM, whether it is diagnosed clinically, with abnormal pneumatic otoscopy and a compatible clinical syndrome (fever, earache, irritability, poor sleeping or eating, respiratory or gastrointestinal symptoms), or by RT-PCR for detecting viral RNA in MEF. Influenza vaccination in children enrolled in daycare appears to reduce the diagnosis of AOM by 33–36%

(Heikkinen et al., 1991a; Clements et al., 1995a). Oseltamivir treatment of influenza in children 1–12 years of age appears to be able to reduce the risk of AOM by 40% during the first 28 days of illness (RR 0.60, 95% CI 0.4-0.95). The benefit is even larger in younger children, aged 1–5 years of age, with a 56% reduction in risk of AOM during the first 28 days (RR 0.44, 95% CI (0.26–0.72) (Whitley et al., 2001). The pathogenesis of AOM in influenza could be the result of viral infection of the middle ear, infection of the eustachian tube with resultant occlusion, or infection of the nasopharynx with resultant inflammation which produces eustachian tube dysfunction. Additional studies will need to be done to further understand the pathogenesis of influenza induced AOM.

Influenza can result in sinus and middle ear involvement and the clinical studies suggest that the influenza vaccine may be especially useful for 'otitis prone' children and that specific antiviral therapy may be beneficial in preventing otitis media in healthy, as well as 'otitis prone' children. An area that needs further study is the effect of specific antiviral therapy on the insertion of PE tubes and adenoidectomy. There are about 1 million bilateral PE tubes placed and 86000 adenoids without tonsillectomy performed in children each year (Paradise, 1977; Bear, 1977). Drugs that could reduce the use of these procedures could result in large cost savings and deserve further investigation.

8.6. Status of RSV fusion inhibitors (Dan Pevear, ViroPharma, Exton, PA)

The only currently approved therapy for RSV is ribavirin, which has limited activity and substantial toxicity. ViroPharma's VP14637 is a novel anti-RSV fusion inhibitor that is 40 000-fold more potent than ribavirin in viral cytopathic effect (CPE) assays against RSV. VP14637 inhibits the early fusion event, as documented by time of drug addition studies, by interacting with a conserved pneumoviral target on the fusion protein (Zhao et al., 2000) VP14637 is highly potent against RSV-A and B strains with an EC_{50} of 0.0005 μM in viral CPE assays. VP14637 is specific for RSV and does not inhibit the replication of other common viral respiratory pathogens. Clinical isolates of RSV collected over the past 10 years from North America and Europe were inhibited at VP14637 concentrations of 0.0001–0.08 μM. It markedly inhibits both virus yields and RSV antigen expression in cell culture. The compound is also efficacious in vivo in the cotton rat model of RSV infection when delivered by small particle aerosol. Escape mutant analysis has identified the conserved fusion protein as the target for VP14637 inhibition. Initial clinical studies with VP14637 are progressing. Several other RSV fusion inhibitors are currently being developed by Wyeth-Ayerst, Janssen Pharmaceuticals, Trimeris and Bristol Meyers Squibb.

8.7. Capsid-binders (Frederick Hayden, University of Virginia, Charlottesville, VA)

Pleconaril is a novel capsid binder with broad anti-picornaviral activity. The drug intercalates into the hydrophobic pocket of the VP1 protein and inhibits attachment and/or uncoating of the virus. The absolute bioavailability is unclear, but oral pleconaril shows dose-proportionality with increasing plasma concentrations with increasing oral dosing (Abdel-Rahman and Kearns, 1998). Ingestion with food significantly increases oral absorption compared to the fasted state. High fat meals appear to increase the C_{max} and AUC 3-fold, while non-fatty meals appear to increase levels to a lesser extent. Pharmacokinetic studies (Abdel-Rahman and Kearns, 1999; Kearns et al., 1999) have shown the drug to achieve its maximum plasma level in 3 h (range 2–5 h) and have a maximum concentration of 2.2 ± 0.5 μg/ml. The drug is extensively protein bound ($> 99\%$) and exhibits a large volume of distribution (60 l/kg) as the result of its high membrane permeability and lipophilic characteristics (Rhodes and Liu, 2001b). It is metabolized extensively with over 30 metabolites excreted in urine and feces. None of the metabolites appear to have significant antiviral activity. Pleconaril exhibits a bimodal elimination pattern with a short (2–4 h) initial T1/2 accounting for most drug and a prolonged (mean, 180 h) terminal T1/2 for the remainder. There is no significant alterations in pleconaril pharmacoki-

netics with increasing age or gender (Rhodes and Liu, 2001a). There are minimal interactions with the CYP450 isoenzymes in in vitro studies (Rhodes et al., 2001) and no drug interactions have been documented with theophylline (Hincks et al., 2001).

Four recent trials, two phase 2 and two phase 3, of pleconaril treatment of picornaviral respiratory infections have been completed during the past 3 years. The first two studies (Hayden et al., 2000, 1999) enrolled patients over the age of 14 with at least one respiratory symptom and 1 systemic symptom who presented within 36 h of onset of VRI symptoms. As fever was allowed and colds were not specifically sought in these studies, picornavirus PCR was positive in only 42–43% of patients. Patients received pleconaril 400 mg TID for 7 days. The study medication was well tolerated with the most common side effects of diarrhea, nausea, abdominal pain, and headache being only slightly more common in the treated patients than the placebo group. Retrospective analysis of the picornavirus-infected enrollees ($N = 527$) from the two phase 2 studies found that early pleconaril therapy was associated with a statistically significant reduction of illness duration, total symptoms score, number of tissues used, and percent of nights of disturbed sleep. There was a 1.5 day reduction in time to alleviation of symptoms (8.5 vs. 10 days, $P = 0.029$). The two phase 3 studies (Hayden et al., 2001) enrolled patients ($n = 1052$ and 1044) older than 18 years old with symptom duration of less than 24 h who felt they had a cold on the day of presentation, had moderate to severe rhinorrhea plus at least one other respiratory symptom, and did not have a fever. Patients also received pleconaril 400 mg TID for 5 days or placebo. Percentage of patients positive for picornaviruses (62–69%) was substantially higher in these studies. In the intent-to-treat-infected population, the aggregate median time to alleviation of illness was 1 day shorter in the treated patients (6.3 vs. 7.3 days, $P < 0.001$) for the two studies. Improvement was noted within 24 h of starting treatment and in all monitored symptoms (rhinorrhea, cough, sore throat, nasal congestion, malaise, and myalgias). As with the first study, pleconaril was well tolerated with slightly more headache, nausea, and vomiting than placebo. Also of note, there was a small increase in total cholesterol ($+5$ mg/dl for pleconaril vs. -4 mg/dl for placebo) and platelet counts ($+15 \times 10^3/\text{mm}^3$ vs. $+7 \times 10^3/\text{mm}^3$) in the treated arm at the end of treatment compared to baseline.

Pleconaril is a generally well tolerated medication that represents the first effective antiviral therapy for rhinovirus colds. Other studies are in progress or planned to assess its therapeutic value in children and patients at increased risk of rhinovirus complications (e.g. asthma, COPD) and its prophylactic efficacy. It is currently under review by the Food and Drug Administration for possible approval for the treatment of picornaviral respiratory infections in patients aged 18 years and above.

8.8. Picornaviral protease inhibitors (Amy Patick, Agouron Pharmaceuticals, San Diego, CA)

As the search for drugs active against picornaviruses continues, the 3C protease (Matthews et al., 1994) was identified as a important target because it lacks similarity to human proteases, is essential for replication, and has a highly conserved active site. Ruprintrivir (AG7088) is currently being investigated as a highly potent (mean EC90 for 48 tested serotypes = 82 nM), peptidomimetic, irreversible 3C protease inhibitor (Dragovich et al., 1998a,b, 1999a,b). Against the same 48 viruses, ruprintrivir was 15- to 150-fold more potent in inhibiting 80% of the virus strains tested when compared to pleconaril and pirodavir while maintaining activity against a broad spectrum of a diverse panel other related picornaviruses.

Recently, a challenge experiment was undertaken in which subjects were inoculated with HRV 39 or Hanks and treated with placebo or ruprintrivir 8 mg intranasally 6 h prior to infection for the prophylaxis arm or 24 h after challenge for the treatment arm. Volunteers given prophylaxis received the study drug either twice or five times a day, while all treatment arm subjects received the study drug five times a day. In the prophylaxis study, intranasal ruprintrivir did

not prevent experimental rhinovirus infection but moderated illness frequency and severity when initiated before rhinovirus exposure. Early treatment also resulted in viral titers, done by either culture or RT-PCR, in ruprintrivir subjects with more consistent and more rapid declines relative to placebo. The medication was well tolerated without significant adverse events noted.

As a result of its performance in an experimental human rhinovirus infection, intranasal rupritrivir was tested in 868 patients presenting with within 36 h of onset of cold-like symptoms. This study failed to find significant differences in mean respiratory or total symptom scores at day 1–5 or in the time to resolution of respiratory or cold symptoms between ruprintrivir and placebo. However, only 27% of enrollees were picornavirus positive. Retrospective analysis suggested that symptoms were reduced in picornavirus-positive patients who were enrolled within 24 h of symptom onset. Future studies of this drug will focus on clinical trial designs that will measure antiviral activity and clinical benefit when started at an earlier time point and to search for alternative intranasal formulations with potential for more effective delivery to the site of action.

Most recently, a class of orally bioavailable, pyridone 3C protease inhibitors have been developed. Further studies of these novel 3C protease inhibitors are being planned to determine the potential of these potent and broad-spectrum, anti-picornavirus antivirals in clinical practice.

9. Summary

The IV International Symposium on Respiratory Viral Infections was successful in summarizing the recent advances in respiratory virus research. Advances in the understanding of the epidemiology and pathogenesis of RSV, influenza, parainfluenza, and adenovirus were discussed. It is clear that there is emerging evidence that the 1918 influenza virus is derived from an avian precursor but that it probably adapted to humans soon before the pandemic. The introduction of novel strains of influenza from the region around Hong Kong are concerning and may result in a future pandemic. Influenza vaccine has been proven to be cost-effective in most patient populations. Several studies how shown that respiratory viral infections can be differentiated by clinical presentation and that rapid diagnostic tests are aiding in the diagnostic process. Respiratory viruses have clearly been implicated in the pathogenesis and frequency of AOM. The hMPV has recently been discovered and appears to be a common pathogen in man. Our understanding of this virus is in its infancy and future studies on its epidemiology, pathogenesis, prevention, and management will likely be presented at future meetings.

There have been many advances in the field of respiratory viral vaccinology over the past year. An RSV vaccine has been studied in adults and appears to be safe and immunogenic. A parainfluenzavirus 3 vaccine is also under development and appears to be safe and immunogenic. Influenza vaccines have been found to be cost-beneficial in most patient populations and can therefore be recommended in most patients as long as the vaccine availability permits complete vaccination of high-risk patients. Several intranasal influenza vaccines are either approved or far along in their development.

The past year has been marked by the availability of new antiviral agents. The Flunet® represents a novel dimer of zanamivir that has pharmacokinetic properties in animals that suggests it can be administered once-a-week. Several RSV fusion inhibitors, such as VP14637, have been developed. This class of agents is highly potent against both RSV-A and B strains and is currently undergoing early clinical trials. Two new classes of anti-picornavirus agents has been tested. The capsid-binder pleconaril is well tolerated and effective therapy of rhinovirus colds. The picornavirus protease inhibitor ruprintrivir is currently being investigated as a highly potent, peptidomimetic, irreversible 3C protease inhibitor. The drug performed well in experimental human rhinovirus infections but did not result in significant differences in the treatment of natural infections. It is still being investigated in different settings and with novel formulations.

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