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Antiviral Research 62 (2004) 75–110
Antiviral Research
www.elsevier.com/locate/antiviral

Conference report

Current research on respiratory viral infections: Fifth International Symposium☆

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Received 8 December 2003; accepted 31 December 2003

1. Introduction

The Fifth International Symposium on Respiratory Viral Infections was convened by The Macrae Group (New York, NY) in Casa de Campo, Dominican Republic on 5–8 December 2002. 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 and summaries of four previous meetings have been published in *Antiviral Research* (Ison et al., 2002; 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.

2. Keynote address: influenza: a natural or man-made bioterrorism agent (Robert Webster, St. Jude Children's Research Hospital, Memphis, TN)

Influenza is caused by a negative sense RNA virus with a segmented genome. Its replication results in numerous errors because of a lack of proof reading mechanisms that, in turn, results in the production of quasi-species in most infected individuals. As a consequence of these errors, there is incredible genetic variability that results in continuous antigenic drifts and intermittent antigenic shifts. These shifts have resulted in the introduction of new strains of virus that often resulted in a pandemic (see Table 1). Inter-pandemic influenza is responsible for over 20,000 deaths per year in addition to significant morbidity and loss of work (Thompson et al., 2003).

The most important pandemic of the past century was the Spanish Influenza pandemic of 1918. The virus infected 28% of all Americans and resulted in 675,000 deaths in the US alone. The death rate among young adults, aged 15–34 years, was 20 times higher than in previous years and depressed the life expectancy of Americans by more than 10 years. The pace and severity of the pandemic is best documented in its history in New Zealand. On 12 October 1918, several members on board the SS Niagara became ill with influenza. Since the boat was carrying Prime Minister Massey, the Minister of Health failed to enforce a quarantine on the boat. As a result, influenza infected much of the city of Auckland, such that by the first week in November it was dubbed the "City of the Dead" and had resulting breakdown of civil services. By November 12, there were two full death trains per day to carry the dead out of the city. Like the US, the mortality rate was highest among people aged 20–44 years. Interestingly, the Maori had a higher death rate (43.3/1000) than Europeans (7.6/1000), a finding that suggests prior exposure to influenza may have minimized mortality. Jeffrey Taubenberger has sequenced the HA, NA, NS, M, and NP genes of the 1918 Spanish influenza virus and found that it is closely related to swine H1N1. Unfortunately, the molecular basis of the high pathogenicity of this virus is still unknown. Likewise, it is unclear if the virus, the hemagglutinin and neuraminidase genes of which derived

☆ The Scientific Advisory Board for the meeting included Frederick Hayden (University of Virginia) and Brian Murphy (National Institute of Allergy and Infectious Diseases, National Institutes of Health), the co-chairs of the meeting and Larry Anderson (CDC), Robert Couch (Baylor College of Medicine), Sebastian Johnston (National Heart and Lung Institute, Imperial College), Yoshihiro Kawaoka (Universities of Wisconsin and Tokyo), Linda Lambert (NIAID, NIH), Peter Openshaw (Imperial College London, St. Mary's Campus), and Albert Osterhaus (Erasmus University). Agouron Pharmaceuticals, Inc., Pfizer Co., Aventis Pasteur, F. Hoffmann-La Roche Ltd., MedImmune, Inc., Solvay Pharmaceuticals B.V., ViroPharma Inc., Wyeth Vaccines and The Macrae Group provided educational grants for this meeting.

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0166-3542/$ – see front matter
doi:10.1016/j.antiviral.2003.12.006

Table 1
Major influenza A viruses of this century

Virus	Circulation	Human–avian reassortment	Attributable deaths first year of circulation
Spanish Flu (H1N1)	1918–1956	No	>20 million
Asian (H2N2)	1957–1967	Yes	>70,000
Hong Kong (H3N2)	1968–present	Yes	>34,000
Russian (H1N1)	1977–present	No	

from an avian influenza virus, directly infected man and then infected pigs or if pigs served as an intermediate host.

Studies of more recent viruses has demonstrated that aquatic birds are the natural reservoirs of all influenza A viruses in other species. In these wild aquatic birds, influenza virus replicates predominately in the intestinal tract and transmission via the fecal–oral route, often through water. There are a limited number of host-specific lineages of influenza viruses in birds and there is a geographical separation into Eurasian and American lineages. Influenza viruses in their natural avian reservoir are in evolutionary stasis, but rapid evolution occurs after transmission to new hosts.

It is felt that swine are significant intermediate hosts as they are susceptible to all influenza A subtypes and have receptors for both avian and mammalian influenza viruses. Acquisition of avian influenza genes by swine influenza viruses has been documented in H1N1 isolates in Europe, H4N6 in Canada, and H9N2 in China, although these latter two instances were transitory. In the United States, H3N2 viruses became a problem in pigs in 1998 when the infection was associated with respiratory signs, high fever (104–107 °F), high morbidity (100%), and high risk of abortion. The responsible viruses were highly transmissible and resulted in variable mortality. Interestingly, analysis of three of the porcine H3N2 viruses revealed that the virus has undergone triple reassortment and had acquired human PB1, HA, and NA genes, porcine NP, M, and NS genes, and avian PA and PB2 genes. There is growing evidence, too, that H1N1 swine influenza A viruses are acquiring avian polymerase genes as well.

Although pigs appear to be an important reservoir and mixing pot for potential human influenza viruses, the chicken H5N1 and H9N2 viruses that have recently emerged in Asia suggest chickens may also be an important intermediary. In 1997, a reassortant H5N1 virus containing H5 Goose/Guangdong/1/96-like HA and other genes from H9N2 Quail/HK/G1/97-like and H6N1 duck/HK/W312/97-like viruses began circulating in chickens. The virus was transmitted to 18 humans and resulted in six deaths. Patients who became infected had high fevers, nausea, vomiting, diarrhea, and pneumonia in addition to alterations in hepatic and renal function. Fortunately there was no human-to-human transmission and the outbreak was halted by the massive slaughter of all poultry in the region. Additional studies of the local chicken market demonstrated that when H5N1 virus infected a chicken it resulted in its death, while chickens co-infected with H9N2 viruses were protected from illness but efficiently shed H5N1. The H5N1 virus still appears to be a problem with re-emergence of a different H5N1 virus in 2001; fortunately an outbreak of human infections was prevented by the massive slaughter of chickens again. Unfortunately, multiple different lineages of H5N1 viruses, all with the Goose/Guangdong HA are still present in local chickens. [Editorial note: In February 2003 infections with an avian influenza A/H5N1 virus resulted in two deaths in a Hong Kong family that visited southern China. During the current influenza season, avian influenza A/H5N1 has again caused disease and death throughout Asia.]

Upon further investigation of these H5N1 outbreaks in chickens and humans, it became clear that quail might play a significant role in interspecies transmission of influenza viruses. It appears that quail acquire infection from fecal sources and then replicate virus in their respiratory tract with evidence of tracheal shedding of virus. The outbreaks in chickens were associated with the reintroduction of H6N1 and H9N2-infected quail into the markets. To address the concerns that quail are intermediates in the transmission of influenza, quail have been banned from all poultry markets in Hong Kong. In addition, ducks and geese, which have been recognized in the past as reservoirs for influenza viruses, are imported separately from all other poultry and are killed and sold chilled instead of the previous practice of selling them live. Finally, all markets are emptied and cleaned monthly with the hopes of reducing transmission of influenza among the poultry.

Since the H5N1 virus has caused severe disease in humans already and is a potential pandemic threat, several researchers are looking for its virulence factors. Clinical experience has documented that H5N1 virus kills all chickens it infects, unless there is coinfection with H9N2 viruses. Likewise, the virus is neurovirulent in mice and ferrets. In humans, mortality is high and post-mortem examination in two individuals found very high cytokine levels. Several studies subsequently documented that the NS1 protein of the H5N1 was an antagonist of the antiviral activity of several cytokines (Seo et al., 2002). Genetic comparison of the NS1 gene of H5N1 viruses from 1997 and 2001 and other human, avian, swine, and equine viruses identified a single Asp92Glu mutation in the virulent H5N1 viruses. Using reverse genetics, it was found that infection due to the viruses with the mutation resulted in greater viral shedding and symptoms in pigs and lacked inhibition of replication typically induced by cytokines in vitro. Furthermore, in-vitro infection of human primary macrophages with H5N1/97 virus up-regulated expression of mRNA encoding TNF-α, IFN-β,

IL-1β, MCP-1, MIP-1α/β, RANTES, IFN-α, and IL-12 and caused increased elaboration of pro-inflammatory cytokines.

As our understanding of current and previous pandemic viruses expands, so do our techniques to learn about the virus and its pathogenesis. One of the major advances is the multi-plasmid reverse genetics system for influenza. This system allows scientists to make influenza viruses to order, to rapidly prepare high growth reassortants for vaccines, to rapidly prepare subtype-specific vaccines for pandemic preparedness, and to better understand the molecular basis of virulence and host range of the virus. Unfortunately, powerful technique brings several challenges. Since it is so easy to make reassortant viruses, it could be possible to create viruses with several pathogenic genes or viruses that are highly resistant to antivirals resulting in highly transmissible and lethal agents that could be used as agents of bioterrorism. In this regard, dangerous constructs need to be contained within BSL3+ level facilities, limiting the number of centers that can study them.

To prepare for the future, with the current risks of bioterrorism and pandemic influenza, it is essential that surveillance, using global WHO human and animal networks, and yet to be developed biosensors, be expanded. Additionally, vaccines and their production need to be studied since it currently takes 6 months to prepare a new vaccine and there may not be a mild warning wave of infection before the next pandemic. Lastly, antivirals need to be stockpiled and new antiviral agents need to be developed.

3. Impact and epidemiology

3.1. Metapneumovirus infections: the new kid on the block (Albert Osterhaus, Erasmus University, Rotterdam, The Netherlands)

Human metapneumovirus (hMPV) was discovered and described by the group at Erasmus University in 2001 (van den Hoogen et al., 2001, 2001). Since then, serologic studies have documented infection in patients throughout the world, including the Dutch Antilles, Barbados, Slovakia, Finland, The Netherlands, Sudan, Somalia, India, and Vietnam (Boivin et al., 2002; Chan et al., 2002; Falsey et al., 2003; Freymouth et al., 2003; Howe, 2002; Jartti et al., 2002a; Nissen et al., 2002; Pelletier et al., 2002a,b; Peret et al., 2002; Stockton et al., 2002). Most children first acquire the infection during the first 5 years of life with infection documented in over 50% of infants in the first year of life. Although most infections have been documented in children, up to 20% of infections occur in patients over the age of 60. Preliminary data suggests that hMPV may be responsible for approximately 7–8% of viral respiratory tract illnesses (RTI) in children and 2–3% of RTIs in adults.

Among the primates, rhesus monkeys (71%) and chimps (59%) frequently have antibodies to hMPV, while they are less frequently found in orangutans (33%) and gibbons (13%). Experimental challenge studies were done in cynomolgus macaques, guinea pigs, ferrets, chickens and turkeys; birds failed to become infected and only the macaques developed disease with evidence of histopathological changes in tissues from the nose to the bronchioles. Repeated infection of the macaques resulted in shorter duration of shedding with subsequent infections. Serum IgA antibodies failed to be detectable until after a second infection; secondary infection resulted in a low level of hMPV antibody production but failed to induce heterologus antibodies against the related avian pneumovirus (APV).

Sequencing of the N, P, M, F, M2, SH, G, and L genes indicates that hMPV is most closely related to APV (van den Hoogen et al., 2002). Hydrophilicity plots of the F revealed two hydrophobic regions with similarity to APV and RSV. The G protein has five glycosylation sites. There appear to be two genetic and serotypic clusters of hMPV with two subclusters of each cluster (A1, A2, B1, B2).

3.2. The new human metapneumovirus as a cause of severe acute respiratory tract infection in hospitalized children: a prospective study (Guy Boivin, Laval University, Québec, Canada)

A new paramyxovirus, the human metapneumovirus (hMPV), has been recently isolated from Dutch children with respiratory symptoms (van den Hoogen et al., 2001). However, the prevalence, clinical presentation, and severity of hMPV infections in children have not been well studied. A multiplex real-time PCR assay was used to detect viral pathogens (influenza A, influenza B, respiratory syncytial virus (RSV), and hMPV) in nasopharyngeal aspirates of hospitalized children (0–3 years) with acute respiratory tract infections (ARTI) during then 2001–2002 winter and spring seasons. Children hospitalized for surgery without respiratory symptoms were used as controls. hMPV was detected in 5.8% of the 208 children hospitalized for ARTI, compared to 51% for RSV and 21.6% for influenza A; no virus was detected in 29.8% of symptomatic patients and 6.8% had more than one pathogen including one case of coinfection with hMPV and influenza A. None of the viruses were detected in 51 controls. Most RSV and influenza infections occurred in January–February, whereas peak hMPV activity was seen in March–April 2002. Overall, the clinical presentation and complications associated with hMPV infection were very similar to those caused by RSV (fever 67% versus 57%, cough 100% versus 99%, rhinorrhea 92% versus 91%, and wheeze 92% versus 95% for hMPV and RSV, respectively). hMPV was associated with bronchiolitis in 66.7%, otitis media in 50%, and pneumonia in 16.7% of infected children; no hMPV children were sick enough to require admission to an intensive care unit. Mean hospital stays were 5.5, 5.8, and 5.0 days for children infected with hMPV, RSV, and influenza A. Although severe hMPV infections are less frequent than those caused by hRSV, the clinical presentation of the two viral

pathogens is virtually identical. Dr. Boivin also discussed an outbreak investigation in which there appears to be a nosocomial outbreak of hMPV in a nursing home, giving further information about the impact of this new virus. In summary, hMPV is a true respiratory pathogen associated with significant morbidity in children.

3.3. Dual respiratory viral infections: two worse than one? (Robert Couch, Baylor College of Medicine, Houston, TX)

Multiple respiratory viruses can be detected sometimes in patients with respiratory tract illness. In various studies, the frequency of dual infection has ranged from 0 to 40% of virus-infected patients, with most studies documenting dual infections in 0–10% of patients. The frequency of dual infection appears to be higher in children than adults. The types of diagnostic techniques utilized greatly impacts the detection of viruses. In a study of 4336 adult patients, many with underlying lung disease, who developed 1341 respiratory infections, dual infection was documented in 1.9% of patients when cell culture alone was used, 8.1% when serology was added to culture, and 11.6% when RT–PCR was added to the previous two techniques (Drews et al., 1997). The most frequently isolated viruses from patients with dual infections included picornaviruses ($n = 33$), influenza A (28), coronaviruses (22), RSV (21), adenoviruses (12), parainfluenza viruses (12), and influenza B (7). The three most common combinations were picornaviruses with influenza A ($n = 10$), cornoavirus (10), and adenovirus (7) (Drews et al., 1997). During the question and answer period, Sebastian Johnston reported that in his study of respiratory viral illnesses in children with a history of wheezing: 15% of patients had dual infections and 5% with triple infections during exacerbations.

The impact of dual infections on disease course has also been studied. In volunteers who were co-inoculated with HRV 15 and another picornavirus (simultaneous infection) or infected with a picornavirus approximately 2 weeks after initial HRV 15 infection (sequential infection), those infected with two viruses simultaneously had no difference in virus shedding or disease severity, whereas those who had sequential infection had reduced virus shedding and disease severity, in comparison to volunteers only infected with HRV15 (Fleet et al., 1965). The exact mechanism for this reduction of viral shedding and symptom severity is not known but transient immunity from the previous infection may contribute to the effect. In children presenting with lower respiratory illness with RSV alone or in combination with another respiratory virus, some studies did not find differences in the severity or duration of the illness, but two studies documented increased severity of symptoms and one documented increased duration of illness (Andreoletti et al., 2000; Cate et al., 1964; Meissner et al., 1984; Papadopoulos et al., 2002a; Portnoy et al., 1966; Ray et al., 1993; Smith et al., 1980; Subbarao et al., 1989; Tristram et al., 1988; Tsai et al., 2001). Preliminary data from Dr. Johnston's study also documented a clear increase in severity of the asthma exacerbation with increasing number of infecting viruses. Although not well controlled for in these studies, the timing of infection and the type of viruses likely impact the duration and severity of illness. Available information is insufficient for firm conclusions on the illness consequences of dual respiratory viral infections, and future studies should be address the relationships between illness severity and etiology, time course, and quantitative virology of dual infections.

3.4. Respiratory pathogens in the first year of life: a birth cohort study using PCR (Sebastian Johnston, Imperial College London, UK)

Numerous studies have documented an association between asthma exacerbations and respiratory viral illness (RVI). Approximately 85% of children and 60% of adults presenting with asthma exacerbations have detectable virus at the time of their exacerbation. Several recent studies have expanded on our understanding of the epidemiology of RVIs and their relationship to asthma exacerbations. In unpublished studies, the presenter's group performed viral culture and PCR for RSV and human rhinoviruses (hRV) on nasal aspirates from 80 children aged 0–16 years who were hospitalized with upper respiratory tract illness (URTI) and wheezing. Ninety-six percent of children under 2 years of age were positive for one of the two viruses (80% RSV, 40% rhinovirus, 20% dual infection), while 80% of children 2 years and older had detectable virus (66% rhinovirus). These results confirm the high frequency of RVI in such patients and the age-related changes in pathogen distribution observed in earlier studies with predominance of rhinovirus infections after age 2 years.

A second study collected nasal aspirates within 24 h of admission among 118 infants admitted with bronchiolitis. Seventy-three percent of these patients had virus detected by PCR (72% RSV, 29% hRV, 9% adenovirus, 3% influenza, 3% parainfluenza, and 3% coronavirus). Dual infection was documented in 19.5% of the patients. Patients with RSV infection tended to be younger (3.2 months versus 5.2 months, $P = 0.005$) and with a higher birth weight than those with hRV infection. Patients with hRV infections alone were hospitalized earlier in an illness episode (1.8 days versus 3.1 days, $P = 0.001$) and had a higher likelihood of having a clinical severity score on admission above the 50th percentile (OR 4.9, $P = 0.022$) after controlling for age, gender, birth weight, presence of fever, and day of disease on admission. Patients with dual infections were hospitalized later in illness than those with RSV or hRV alone (4.7 days versus 3.1 days ($P = 0.028$) versus 1.8 days ($P = 0.001$)), consistent with the interpretation of dual infection being a sequential event (Papadopoulos et al., 2002b).

Another recent unpublished epidemiologic study, the Western Australian Childhood Asthma study, conducted by Drs. Peter Sly, Patrick Holt, and Merci Kusel, enrolled 263 newly born infants who had at least one parent with

physician-diagnosed atopic disease. All acute respiratory illnesses were recorded during the first year of life and home visits by a study nurse were arranged within 24 h of onset of illness for assessment of symptoms and collection of nasal aspirates. Two control visits were done when the child was asymptomatic. During the first 6 months of life and subsequent 6 months, 59 and 86% of the children, respectively, developed an upper respiratory tract illness (URI). Non-wheezy lower respiratory tract illness (LRI) developed in 28 and 52% of children during the first and subsequent 6 months, respectively, and wheezy LRIs developed in 10 and 26%, respectively. Among the first 656 samples from July 1996 to February 1998 (369 ARI, 260 controls), 19% of the controls were positive (11% picornavirus, 8% RSV) compared to 65% of the ARI samples (55% picornavirus, 10% RSV, and 3% with dual infection). Among those with URI, 84% had picornaviruses, 11% had RSV, and 5% had dual infections; non-wheezing LRI was associated with picornavirus detection in 74%, RSV in 23%, and dual infection in 3%; wheezing LRI was associated with picornavirus in 70% of cases, RSV in 22%, and dual infection in 8%. The ratio of URI to LRI was 1.9:1 for picornavirus and 1:1 for RSV. Although RSV is perhaps more likely to be associated with severe lower respiratory tract illness, these data indicate that rhinoviruses are major respiratory pathogens during the first year of life and cause about three times as many LRIs in the first year of life as RSV in non-hospitalized infants. The specific picornavirus that cause these illnesses remain to be identified. Further PCR tests for coronaviruses, influenza A and B, parainfluenza viruses 1–3, adenoviruses, hMPV, *Mycoplasma pneumonia*, and *Chlamydia pneumoniae* are in progress.

3.5. Molecular epidemiology of adenovirus type 7 respiratory infections (Adriana E. Kajon, Lovelace Respiratory Research Institute, Albuquerque, NM)

The adenoviruses constitute an important group of human pathogens with a capacity to cause a wide range of illnesses in man. Adenoviruses are responsible for 5–10% of the cases of pediatric acute respiratory infections (ARI) that require hospitalization. Nosocomial outbreaks of adenovirus respiratory infection are frequently reported. The adenovirus virion consists of a non-enveloped icosohedral capsid enclosing a double-stranded DNA genome of approximately 36,000 bp. The human adenovirus family currently comprises 51 serotypes that are classified into six species (formerly subgenera), A–F, based on their genomic characteristics, tropism, associated disease and other biological properties. Respiratory disease is mainly caused by adenovirus serotypes classified within subspecies B1 (Ad3, Ad7, Ad16, Ad21, Ad50), species C (Ad1, Ad2, Ad5, Ad6), and species E (Ad4). Among the most prevalent serotypes associated with human respiratory disease, adenovirus type 7 (Ad7) is a frequent cause of severe clinical presentations in children and young adults, in whom it causes epidemics of acute febrile respiratory illness among military recruits (Ryan et al., 2002). Extrapulmonary manifestations, rare fatalities, and residual lung damage are observed in association with Ad7 infection (Becroft, 1967; Steen-Johnsen et al., 1969). Affected patients surviving severe disease may have chronic sequelae in the form of bronchiectasis, bronchiolitis obliterans and hyperlucent lung (Murtagh and Kajon, 1997).

Restriction enzyme analysis of Ad7 strains has shown the existence of considerable intra-serotypic genetic variability. Over the last two decades reports of molecular epidemiological studies of Ad7 infection have shown that certain genome types predominate in an area for long periods of time and then are replaced by a new genome type. The molecular bases of these substitutions of one genome type for another, called genome type “shifts”, and the selective forces driving adenovirus evolution in nature are unknown. Moreover, the impact of genetic variability on adenovirus virulence is poorly understood. A shift from Ad7c to Ad7b occurred in Europe in 1969 and in Australia in 1975 (Wadell et al., 1985). A shift from Ad7b to Ad7d occurred in China between 1965 and 1980 (Li et al., 1996), and a shift from Ad7c to Ad7h took place in the South America (Argentina, Chile and Uruguay) in 1986 (Kajon and Wadell, 1994). Ad7b is still the most prevalent genome type isolated in Europe and North America and Ad7h is still recovered from the nasopharyngeal secretions of children hospitalized for ARI in the South America, observations suggesting an adaptive advantage of their array of genes over the preceding prevalent genome type in the region. Interestingly, Ad7h has been recently detected in the United States and Japan in association with respiratory illness (Erdman et al., 2002) and in Brazil in association with acute conjunctivitis, findings that suggest a recent introduction and dissemination of this virus from previously geographically restricted areas of circulation. Ad7d$_2$, originally reported to circulate in China and later on in Japan and Israel, has been identified in recent outbreaks of ARI in the United States affecting children and military trainees (Erdman et al., 2002; Gerber et al., 2001).

The major neutralizing epitopes of different Ad7 genome types, the hypervariable regions of the hexon, exhibit a high degree of conservation at the amino acid level with the exception of Ad7p and Ad7d (Li and Wadell, 1999). These findings suggest that genome type shifts are not strongly determined by selection of antigenic variants. We have initiated the study of genetic variability in regions of the viral genome encoding immunomodulatory functions (Horwitz, 2001) and have identified sequence differences in the E3 region among various Ad7 genome types. The occurrence of intermediate variants that have a 7-like hexon gene and a 3-like fiber gene has also been detected (Kajon and Wadell, 1996b).

However, the implications of genetic variation for human disease are still unclear and will remain elusive until Ad7 genome types are characterized phenotypically and a correlation between genotype and phenotype is established.

4. Virology

4.1. Mechanisms of influenza virulence (Yoshihiro Kawaoka, Universities of Wisconsin and Tokyo, USA and Japan)

The Spanish Flu pandemic of 1918 resulted in significant morbidity and mortality in part because of the high rate of viral pneumonias and lower respiratory tract complications. Recently, certain genes of the virus have been sequenced to help determine those responsible for its virulence. Reverse genetics has been used to create viruses with an influenza A/WSN background, that contain genes from the 1918 strain or more contemporary strains. In a recent experiment, conducted in a BSL4 laboratory, three viruses were created: a wild-type WSN virus, a WSN virus with 1918 HA and NA genes, and a WSN virus with 2001 HA and NA genes. In mice the LD_{50} for the 2001 HA/NA virus was $>10^5$, compared to $10^{3.1}$ for both the wild type and 1918 HA/NA viruses. Lung titers of infected mice were higher in the wild-type ($10^{6.4}$) and the 1918 HA/NA viruses ($10^{6.1}$) than in the 2001 HA/NA virus ($10^{4.1}$). The degree of lung inflammation was most severe, with evidence of significant hemorrhage, in mice infected with the 1918 HA/NA virus and least with the 2001 HA/NA virus. Viral antigen was distributed differently in the lungs for the three viruses at day 6. The WSN wild-type virus was predominant in the bronchioles; the 1918 HA/NA virus had evidence of diffuse involvement of the pulmonary parenchyma; the 2001 HA/NA virus had minimal staining of the lower airways. Lastly, another recombinant virus was constructed with the A/Memphis/8/88 (H3N2) background. The wild-type virus was non-lethal in mice, but the virus with 1918 HA and NA genes was uniformly lethal. Such findings indicate that HA and NA of the 1918 virus are linked in part to its virulence in mammalian hosts.

Protection against the 1918 virus could be achieved by antivirals and antibodies. The currently available anti-influenza agents have excellent activity against the 1918 strain of influenza A responsible for the pandemic (Tumpey et al., 2002). Antibodies have been tested in residual blood samples of patients before and after 1918. High levels of antibodies directed against the 1918 HA are present in blood in patients born before 1918 but generally not after. The concept of original antigenic sin, in which humans exposed or vaccinated to influenza will produce higher antibodies against the first virus they were exposed to than against subsequent viruses, may explain this finding. In turn, this could partly explain why older patients may have been relatively protected relative to younger individuals during the 1918 pandemic.

Another question that has recently been answered, although the significance of it is still under investigation, is how the virus is packaged. Analysis of the genes of influenza documented that all 8 segments of influenza A have conserved, non-coding regions at both ends. Several studies were then conducted to determine if segment incorporation was random or selective. First, viruses with 6, 7, and 8 segments were created to assess the efficiency of virion production. In cell culture, virus replication was greatest when all 8 segments were present and least when only 6 were present. Next, reverse genetics was used to create two viruses, one had the NA gene replaced with the gene for green florescent protein (GFP) with the conserved non-coding flanking base pairs (NCFBP) before and after (NCFBP–GFP–NCFBP) and another in which the GFP was flanked by NCFBP and the NA gene (NCFBP–NA–GFP–NA–NCFBP). In the GFP only segment, only 0.1% of infected cells made GFP as compared to 91% of the NA–GFP segment. This suggests that, although the NCFBP is required, the coding region, in this case NA, is required for incorporation into new virions. Additional studies documented that this virion incorporation signal (VIS), which frequently contained UAU coding regions, was unique for each segment. The genes for the HA, NP, and M require VIS at both ends while the NA and NS genes only require a VIS at the 3′ end. Lastly, the packaging of segments within the virion was assessed through electron microscopy, which demonstrated that there is likely an interaction between base pairs on each of the segments that results in the segments aligning with one another in a fixed way (Fujii et al., 2003). These interactions require greater study as this is a potential target for drugs or vaccines of the future.

4.2. Developing live vaccines for human respiratory paramyxoviruses (Peter Collins, NIAID, NIH, Bethesda, MD)

The ability to recover infectious respiratory syncytial virus (RSV) and parainfluenza viruses (PIV) serotypes 1, 2 and 3 from cloned cDNAs, a technique called “reverse genetics”, offers a new approach to developing live attenuated vaccines. The main challenge in creating a potential live vaccine is to develop a virus that is both highly and stability attenuated while maintaining a satisfactory level of immunogenicity. Reverse genetics have be used to achieve this goal by creating an array of potential mutations, fine tuning the level of attenuation, increasing the immunogenicity of the virus, and decreasing its reactogenicity. RSV and the PIVs have modular genome organizations (3′-N-P-M-F-HN-L-5′ for the PIVs and 3′-NS1-NS2-N-P-M-SH-G-F-M2-L-5′ for RSV) and short cis-acting RNA signals that make for viruses that are highly amenable to genetic manipulation. Vaccine candidates for RSV (strain A2 of subgroup A) had previously been developed by conventional biological means, involving passage at suboptimal temperature (“cold passage” [*cp*]) followed by chemical mutagenesis and the identification of temperature sensitive (*ts*) derivatives. Several of these strains were evaluated clinically and one, called *cpts*248/404, was shown to be infectious, immunogenic, and protective against a second vaccine dose in 1–2-month-old infants, the target age for RSV vaccination. However, it retained the ability to cause mild nasal congestion and thus would require further attenuation. This proved

unfeasible by conventional methods. These viruses were sequenced and their mutations were identified and the phenotype conferred by each was confirmed by incorporation into wild type recombinant virus, providing a list of known attenuating mutations. This list was expanded by finding that a number of RSV genes, including NS1, NS2, SH and M2-2, could be deleted to yield derivatives that proved to be attenuated in vivo. This new knowledge was applied to reverse genetics to create two new viruses, 248/404/ΔSH and 248/404/1030/ΔSH, the second of which shows promise in early clinical trials and appears to be free of residual virulence. Other viruses are being developed that contain some of the more highly-attenuating gene deletions, or which contain various combinations of gene deletions and point mutations. Reverse genetics also can be used to increase gene stability to prevent reversion by incorporating multiple point mutations, by designing amino acid substitutions to involve at least two nucleotide changes compared to assignments yielding wild type or wild type-like phenotypes, and by employing gene deletions. Further investigation suggests that moving the G and F genes to a more proximal location in the genome results in increased efficiency of G and F expression and enhanced immunogenicity. Likewise, deletion of the M2-2 ORF, a putative regulatory factor, resulted in a shift in RNA synthesis from RNA replication to transcription, with concomitant increased antigen expression. It might also be possible to reduce the reactogenicity of an RSV vaccine using strategies developed from immunobiological studies: one example would be to ablate the secreted form of G, which has been implicated in preferential sensitization of Th2 T lymphocytes. Reverse genetics also was used to create RSV subgroup B vaccine candidates by replacing the G and F protective antigen genes of RSV subgroup A vaccine candidates with their subgroup B G and F counterparts.

Reverse genetics has also been applied to hPIV3 to develop vaccine candidates. A promising hPIV3 vaccine candidate, *cp*45, had previously been developed by the conventional approach of cold passage. Nucleotide sequencing identified 15 potential attenuating mutations, which were characterized by reverse genetics: the major attenuating mutations were found to involve three *ts* mutations in L and three non-*ts* mutations in C and F. Reverse genetics was used to develop additional means of attenuation, which included silencing the C, V and D open reading frames as well as the novel strategy of "importing" mutations into hPIV3 from corresponding genes in heterologous viruses using amino acid sequence alignments to identify corresponding residues. Bovine (b)PIV3 represents a completely different vaccine strategy, one that is based on the finding that bPIV3 is antigenically related to hPIV3 but is naturally attenuated in primates. This is a "Jennerian" strategy, analogous to the use of cowpox virus as a vaccine against small pox. This strategy was improved by using reverse genetics to construct a version of bPIV3 in which the F and HN major antigenic determinants were replaced by their hPIV3 counterparts, thus providing for fully homologous immunity against hPIV3. This chimeric virus retained the attenuation phenotype and represents a promising vaccine candidate. The basis for the host range restriction of bPIV3 was investigated by the replacement of individual genes in hPIV3 with their bPIV3 couterpart. This showed that most of the bPIV3 genes made individual contributions to the attenuation phenotype. This suggests that a vaccine based on the attenuated bPIV3 backbone should be very stable. It also indicated that the introduction of individual bPIV3 genes into hPIV3 is an additional strategy for attenuation, one that is now being pursued with a version of hPIV3 containing the bPIV3 N gene. Reverse genetics also is being applied to hPIV1 and hPIV2, for which no live vaccine candidates are presently available and for which there are no attenuated animal virus counterparts as potential Jennerian vaccines. Here, the strategy of "importing" mutations, in particular from RSV and hPIV3, has been particularly useful and has resulted in the development of a number of attenuating mutations that will serve as the basis for live hPIV1 and hPIV2 vaccines.

Finally, reverse genetics have been used to create attenuated, chimeric virus vectors to protect against multiple paramyxoviruses. One multivalent candidate used the above-mentioned bPIV3/hPIV3 chimeric virus as a vector for the RSV F and G genes. This virus thus combines two vaccines into one. Importantly, the PIV3 backbone replicates in cell culture more efficiently and expresses higher levels of protein than RSV, and avoids the physical instability that is characteristic of RSV. This chimeric vaccine exhibited satisfactory levels of attenuation and immunogenicity against hPIV3 and RSV when evaluated in Rhesus monkeys, and will be developed for clinical evaluation. The availability of PIV vectors expressing RSV antigens offers a novel approach for the immunization or, alternatively, provides an efficient method of "boosting" an initial vaccination made by a live RSV virus. It is anticipated that these methods will result in the development of live vaccines against RSV and hPIV3, which preferably would be given in the first weeks of life, and against hPIV1 and hPIV2, which cause disease somewhat later during the first year of life such that vaccination could begin at 6 months of age.

4.3. Ambient temperature specimen collection: evaluation of PCR tests for influenza and adenovirus (Kevin L. Russell, Navel Health Research Center, San Diego, CA)

Infections caused by influenza and adenovirus are not only common among military personnel but also make them less prepared for battle. The current threat of bio-warfare, in addition to availability of anti-influenza drugs for prevention and treatment, makes the rapid identification of these pathogens essential to medical personnel and the military. In the battlefield situation, ambient storage and transport of specimens would be desirable.

The Department of Defense Center for Deployment Health Research, the Naval Health Research Center, the Armed Forces Institute of Pathology, and the Fort Jackson

Army Recruit Training Center have collaborated to assess the performance of PCR assays for influenza and adenovirus of samples stored at ambient temperature in comparison to viral culture using frozen fresh samples. Any army recruit at Fort Jackson, SC who presented with an oral temperature $\geq$100.5 °F and a cough or sore throat during the study period (December 2001–March 2002) had two nasal swabs collected. One was inoculated into viral transport media and then frozen (−70 °C) for later viral culture, while the other was stored in ethanol at ambient temperature.

In samples collected from 195 symptomatic recruits, viral culture was positive in 46% of patients (14% influenza (27/28 H3N2, 1 B), 32% adenovirus (10 typed, all Av4)). No dual infection were observed with culture techniques. PCR detected influenza A in 25% of patients and adenovirus in 47% of patients. There were 12 patients (6%) PCR positive for both adenovirus and influenza. The results confirm the greater sensitivity of nucleic acid detection methods compared to culture under these circumstances. The sensitivity and specificity of the culture, considering the PCR to be the gold standard, were 64 and 96%, respectively, for adenovirus and 49 and 98%, respectively, for influenza A. There was no direct comparison to PCR positivity of samples frozen immediately versus specimens stored at ambient temperature, although these studies are planned for the 2002–2003 season.

Since room temperature storage techniques are easier to accomplish in most locations and since PCR techniques are widely available to the military, the techniques used in this study should expand the ability to provide respiratory pathogen surveillance. Several subsequent studies are currently under way to standardize the methodology and equipment for the military and to provide information on yield of molecular techniques over time after collection of specimens (6–12 months after collection).

5. Pandemic planning

5.1. Pandemic preparedness: World Health Organization perspective (Klaus Stöhr, WHO, Geneva, Switzerland)

Unfortunately, the world does not appear to be prepared for a pandemic of influenza. Thirty-one countries have plans but only four have formalized plans (Slovak Republic and Norway in 2001, New Zealand and Canada in 2002). All of the plans have been based on the WHO phases as outlined on the Global Agenda on Influenza (http://www.who.int/influenza) which outlines four major areas with 17 associated priority activities. Guidelines for vaccines have been developed, but there is widespread concern that vaccine production will lag behind needs. The WHO hopes to increase the awareness of the importance of pandemic planning and the development of national pandemic plans. Likewise, the WHO is hoping to increase utilization of vaccines and antivirals and to develop strategies to acquire and utilize new vaccines and antivirals. Lastly, the WHO is advocating for additional research on pandemic viruses. These efforts are being heard, as a recent G7+ Round meeting to discuss bioterrorism set aside time for discussion of influenza pandemic planning. Although progress is being made, a pandemic may occur at any time and greater steps need to be taken to minimize its potential impact.

5.2. Pandemic preparedness: United States perspective (Nancy Cox, Centers for Disease Control, Atlanta, GA)

The United States Department of Health and Human Services has established the Interagency Pandemic Influenza Working Group, with input from the Centers for Disease Control, the Food and Drug Administration, the National Institutes of Health, the Department of Defense, the Office of the General Council, the Office of Planning and Evaluation, the Office of Emergency Preparedness, and the National Vaccine Advisory Committee. The goals of this Working Group were to develop a plan to limit the burden of disease, minimize social disruption, and reduce economic loss during an influenza pandemic. These goals are to be met through three objectives: improving surveillance, response, and infrastructure. Several actions have been proposed to strengthen national and global capabilities for virologic and disease surveillance with a view to increasing the likelihood of early detection of viruses with pandemic potential. First, the WHO Global Influenza Surveillance network, with 112 sites in 80 countries and 4 collaborating centers in Atlanta, London, Melbourne, and Tokyo will have to be expanded and improved. Increased collaboration between the WHO and CDC in the form of laboratory and epidemiology training has taken place during the past decade. Animal influenza surveillance is also being enhanced through collaborations between NIH, WHO and other partners. CDC has worked with states to expand the Sentinel Physicians Network such that it currently includes over 1400 physicians in 47 states and the District of Columbia. Work is ongoing to establish a system to monitor hospitalized cases of influenza and to further facilitate the transfer of technology via training and reagent distribution. The US Department of Defense surveillance sites also contribute to global influenza surveillance.

The Interagency Pandemic Influenza Working Group is also focused on improving national readiness to respond to a pandemic. The CDC has developed a planning guide for state and local health officials (http://www.cdc.gov/od/nvpo/pandemicflu.htm) that has helped in the development of pandemic planning guides for 47 states, 4 of which are available on the internet. The focus of these plans is to create decision-making protocols for response to a pandemic, to develop flexible contingency plans for procurement and distribution of vaccines and antivirals, to reduce liability risks, and to prioritize groups for receipt of vaccines and/or antivirals. Improved readiness has also

focused on identification of specific resources required for a response, including additional medical personnel, medications and vaccines. A web-based program, FluAid 2.0 (http://www2.cdc.gov/od/fluaid) has been developed which will help states estimate the potential impact of influenza.

The third, and perhaps largest objective of the Working Group, is to strengthen influenza-related public health practices, infrastructure, and research. A successful component of this objective is the promotion of adult immunization programs with improved vaccine coverage of individuals at increased risk of complications from influenza. Several groups are developing communications networks and protocols to disseminate up-to-date information to healthcare providers and the public. Research on influenza biology and epidemiology, animal surveillance, and vaccine and antiviral development has been and will continue to be supported by NIH through grants to academic researchers and through grants that support public–private partnerships. Development of cell culture-based vaccines and the testing in humans of candidate pandemic vaccines made using reverse genetics or classical reassortment techniques should be encouraged. There is also a need to test adjuvanted vaccines and to develop new antiviral agents. It is noteworthy that there are currently only two influenza vaccine manufacturers licensed to distribute vaccine in the US for the 2003–2004 influenza season. It is also noteworthy that 80–90% of produced vaccine is purchased privately, and it takes nearly a full year from the time of selection of strains to the production of usable vaccine. As a result, it will be essential to encourage new vaccine manufacturers and new approaches, including reverse genetics to prepare seed strains, in addition to developing and implementing a vaccine policy that ensures adequate and fair procurement and distribution of the vaccine during an influenza pandemic. Such policies will need to take into account liability protection and equitable global access. Finally, identifying appropriate antiviral drugs to stockpile and developing plans to prioritize use of these antivirals are necessary for pandemic preparedness.

Despite all of the progress made by the Working Group, many obstacles remain that prevent effective implementation of a pandemic preparedness plan. It is essential that policy makers and the public understand the potential threat that influenza poses to mankind so that firm political and financial commitments are made to minimize the impact of a pandemic. The best defense against an influenza pandemic is to strengthen the capacity to respond to seasonal epidemics of influenza through improved surveillance, increased vaccine use, enhanced vaccine production, and development of a more effective vaccine delivery infrastructure that could function effectively in an emergency. Policy makers should take advantage of and integrate bioterrorism planning with pandemic influenza preparedness and develop flexible, science-based policies. By combining all of these efforts we will be better prepared for the next pandemic of influenza.

5.3. Pandemic preparedness: European perspective (Albert Osterhaus, Erasmus University, Rotterdam, The Netherlands)

Despite the significant progress made in some countries, pandemic influenza planning in Europe is still in its infancy. A major obstacle to pandemic planning in Europe is the lack of a centralized planning organization and dependence on national governments to address public health issues. The first pandemic planning meeting of European Union members included policy makers, scientists, and industry in Brussels, Belgium in November 2001. Since then, an EU draft pandemic plan, with guidance from the WHO's draft guidelines and those prepared by the CDC, has been produced and is currently circulating for discussion. In addition, the IVS Taskforce was developed and has two important subcommittees: the Policy, Practice, and Communications committee and the Science, Production, and Regulations (SPR) committee. The SPR committee has developed goals of creating high-yield seeds through production of influenza virus libraries, with the help of MedImmune, CDC, and European FluPlan partners, and enhancement of reverse genetics technology. The SPR is also working to enhance reagent availability, speed regulatory clearance of future vaccines and antivirals, and to collect clinical data on the safety, necessary antigen concentrations, most appropriate adjuvants and dose schedule, and immunogenicity of candidate vaccines.

In addition, it hopes to move studies forward to allow proof of concept of novel vaccine technologies. One study in this direction has looked at the amount of antigen needed and the relative production capacity of vaccine using several different scenarios. Currently, most trivalent vaccines require 15 μg of antigen for each virus and have a production capacity within Europe of 1 million doses per week. If a monovalent vaccine was used in combination with a potent adjuvant, the required antigen could be potentially reduced to 3.25 μg and increase capacity to 12 million doses per week. Two recent meetings have helped to identify candidate vaccines for H5N3, H9N2, and H2N2 viruses, to collect data on enhanced vaccine efficacy with the use of adjuvants, and to develop protocols to fast track registration of vaccines.

A significant issue for Europe is that only a few of the countries have vaccine production capacities, leaving most nations dependent on others to supply their vaccines. It is estimated that there will be a 189 million dose shortfall of vaccine in the EU during a pandemic. Likewise, there has yet to be a concerted effort to stockpile antiviral agents and supplies of these agents are limited.

Three groups are actively working on pandemic influenza issues in Europe currently. The European Scientific Working Group on Influenza has meetings to address pandemic preparedness. The European Influenza Surveillance Scheme has been designed to collect and exchange information on influenza activity throughout Europe and currently encompasses 10,500 physicians and 26 laboratories in 20 countries. Pandemic plans have been developed in 19 of the 20

European Influenza Surveillance Scheme participating countries, although implementation of the plans has been, for the most part, limited. Finally, the European Union has funded the Fifth Framework Program including FluPlan and NovaFlu 2001. These programs hope to address development of novel vaccines and vaccination strategies and formulations.

5.4. Pandemic preparedness: Australian perspective (Alan Hampson, WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia)

Australia has developed a 'Framework' for an influenza pandemic plan and has begun to implement its recommendations. The Influenza Pandemic Planning Committee (IPPC) was chartered to develop a contingency plan and make recommendations for infrastructure and strategies required to minimize the mortality, morbidity, social disruption and economic losses associated with an influenza pandemic. The work of the Committee and its recommendations were published as a comprehensive document which incorporated as an appendix the contingency plan for health care institutes prepared for the Victorian state plan: http://www.health.gov.au/pubhlth/strateg/communic/tech/influenza.htm.

A condensed, action-oriented version of the plan was subsequently adopted by the Communicable Diseases Network as a basis for action. The major recommendations were the maintenance of an ongoing pandemic planning committee, further impact modeling, adoption of WHO 'alerts', improvement of interpandemic use of vaccine, development of an antiviral stockpile, and development of communication strategies. A National Pandemic Action Committee (NIPAC) has recently been formed with the following terms of reference:

During the interpandemic period:

- Assisting the Government with development of national policies on vaccination strategies, surveillance and antiviral stockpiling;
- Establishing working parties to progress policy issues from the Action Plan;
- Collaborating with the States and Territories to prepare their plans;
- Revising and promoting the Action Plan in consideration of the changing global agenda;
- Regularly reporting progress to the Department of Health and Ageing.

Following WHO declaration of a pandemic:

- Initiating the national response and monitor the pandemic;
- Facilitating communications;
- Alerting government regarding urgent issues and advising actions to reduce public health risks;
- Evaluating the pandemic response.

Similarly the Australian states are moving to pandemic action plans. However, whilst these steps have been taken and pandemic planning has been elevated within the bureaucracy, there has yet to be significant commitment of funds, there has been little regional involvement and, apart from representation through the WHO Collaborating Centre, Australia has not yet contributed greatly to global pandemic preparedness.

5.5. Pandemic vaccine supply issues from the manufacturer's perspective: inactivated vaccines (David Fedson, Formerly with Aventis Pasteur, Lyon, France)

The health and economic benefits of influenza vaccination are firmly established. Influenza vaccine use is increasing worldwide, especially in developing countries. In the year 2000, approximately 234 million doses of vaccine were distributed. Of these, 34% of the doses were used in countries outside North America, Western Europe and Australia and New Zealand. Most (85%) of the world's influenza vaccine is produced in eight developed countries located in Western Europe, North America and Australia.

Susceptibility to pandemic influenza will depend on whether the virus is a re-emergent virus similar to one that has infected people in the past, for example influenza A (H2N2), or an entirely new virus like the avian influenza A (H5N1) virus that appeared in Hong Kong in 1997. If it is an influenza A (H2N2)-like virus, people over the age of 35 years (approximately 40% of the world's population) are likely to have been immunologically primed by past infection and will require only one 'booster' dose of vaccine. If the virus is entirely new, no one will have been primed and everyone will probably require two doses. Moreover, because the clinical effectiveness and cost-effectiveness of vaccinating not only older adults but also younger adults and children is widely appreciated, health officials in many countries may choose to vaccinate most, if not all of their populations. Thus the number of doses of vaccine that will be needed for a pandemic will be much larger than the number currently used each year.

In inter-pandemic years, companies sell influenza vaccine through contracts with public health authorities or directly to distributors, institutions, employers or physicians. Prices are low, and the vaccine is generally considered to be a commodity product by companies and health authorities alike. For this reason, companies make conservative estimates of how much vaccine they will produce each year. When demand for vaccine increases suddenly, as it will when a pandemic threat appears, vaccine companies will probably lack the production capacity to respond adequately and quickly.

Because a pandemic vaccine will probably be monovalent rather than trivalent, it is theoretically possible that the number of doses that might be produced could increase three-fold. Nonetheless, pandemic vaccine is still likely to be in short supply in most countries. Prudent national health officials may choose to lock in a future supply of pandemic vaccine by negotiating a long-term forward contract with a vaccine company. Canadian health officials have negotiated

such a contract with Canada's sole domestic producer, but no such contracts have been written in other countries. When a pandemic threat appears, within a period of a few months scores of countries will try to negotiate contracts for pandemic vaccine supply with a small number of companies. The negotiations will be chaotic and, for the most part, fruitless. Moreover, they are likely to be compromised by the 'nationalization' of vaccine production in countries that have influenza vaccine companies. This is what political leaders in the United States did for Swine influenza vaccine production in 1976. Canadian health officials remember this well, which is one reason why they signed their contract with their domestic influenza vaccine producer. Political leaders in countries without vaccine companies will not have this option.

A new approach must be devised that guarantees there will be an adequate supply of pandemic vaccine to meet the level of demand in all countries. This new approach must address the following six major issues.

(1) When a pandemic threat appears, there will be delay in preparing seed strains for vaccine production. This might be overcome by using reverse genetics to prepare these seed strains. In principle, reverse genetics could be used to prepare high-growth variants for efficient production. This may be essential if the pandemic virus is of avian origin because many of these viruses grow poorly (or not at all) in embryonated eggs, the culture system used for all current influenza vaccine production. As long as issues regarding the worker safety and the intellectual property rights attached to reverse genetics can be sorted out beforehand, this promising technique could be the key factor for ensuring adequate production of pandemic vaccine.
(2) Whether the pandemic threat is due to a re-emergent (A/H2-like) or novel (e.g. A/H5-like) virus will determine not only the kind of vaccine that should be produced but also whether one or two doses will be required. Every effort must be made to determine the minimal antigenic content of a dose that is acceptably immunogenic. Studies conducted many years ago indicated that whole cell vaccines were more immunogenic than split virus or subunit preparations of similar antigenic content. Moreover, recent studies have shown that an adjuvanted vaccine will probably be needed for at least the first if not both doses. These studies need to be extended in a larger series of clinical trials of candidate 'pandemic-like' vaccines. The trials must be publicly funded and designed to determine the optimal characteristics of a pandemic vaccine and vaccination schedule.
(3) Current regulatory requirements will delay the registration of pandemic vaccines. In Western Europe, immunogenicity studies must be undertaken by vaccine companies each year in order to register their products. Such trials are costly and time consuming, and they are not required in North America or Australia. Moreover, there is widespread international trade in influenza vaccines in inter-pandemic years. For the next pandemic, such trade is likely to be much more extensive. If pandemic vaccines are to be delivered quickly to the countries where they are needed most, a global protocol will be required that will allow the pandemic vaccine produced by any company to be registered for use in any country of the world. The need for a protocol for the global registration of pandemic vaccines should be considered before the next pandemic threat appears.
(4) Countries will be better prepared for the next pandemic if they can increase their use of influenza vaccine in inter-pandemic years. This was started by the Canadian province of Ontario in 2000, when it extended its recommendation for influenza vaccine to all people over 6 months in age. One reason for instituting this program was to be better prepared for a pandemic. Health officials can help prepare for the next pandemic by expanding their influenza vaccination recommendations and extending their programs for public reimbursement for vaccination in inter-pandemic years.
(5) The traditional market approach for providing inter-pandemic vaccine will not be able to meet the global demand for pandemic vaccine. Although many vaccine companies are expanding their production facilities, it is still difficult for them to estimate future demand for inter-pandemic and pandemic vaccines. This is unlike the situation for pediatric vaccines for which precise estimates of the size of future target populations are well known. One way to overcome uncertainty about future demand for influenza vaccines would be to: (a) obtain country-specific data on the number of doses of influenza vaccine distributed each year by each vaccine company and rapidly report the total number of doses distributed per 1000 population in all countries; and (b) obtain 5-year rolling forecasts of the demand for inter-pandemic and pandemic vaccine from health officials in each country. The availability of such past and future information would challenge health officials to make more comprehensive plans for vaccination programs and help companies make more realistic plans for vaccine production.
(6) Political leaders in most vaccine-producing countries will probably prohibit the export of domestically produced pandemic vaccine to other countries until they are certain their national needs have been met. As a result, countries that do not have vaccine companies, including many that currently use large amounts of vaccine in inter-pandemic years, may find it difficult or impossible to obtain supplies of pandemic vaccine. To prevent this from happening, international political agreements must be negotiated beforehand that ensure adequate production and equitable distribution of pandemic vaccines to both vaccine-producing and non-producing countries. The European Commission has begun doing this for Europe. Something similar needs to be done for the entire world.

Effective planning for pandemic influenza and the global vaccine supply will require the skills of experts in virology, epidemiology and vaccination. Equally important, however, will be the skills of experts in politics, economics and law. Coordinating their efforts at an international level will be very difficult. That the task must be undertaken cannot be questioned. Because the health benefits of its success are certain, the challenge it presents is extraordinarily compelling.

5.6. How good are the rapid diagnostic tests really? (Peter Wright, Vanderbilt University Medical Center, Nashville, TN and Ruth Karron, Johns Hopkins University, Baltimore, MD)

Rapid diagnostic tests for respiratory viral illness has become an increasingly important tool because of the advent of effective antiviral agents that are maximally effective if started early in the course of the illness, the need to isolate patients who could pass their infection to other ill patients, and the need to differentiate between these viral illnesses and other respiratory tract pathogens, including bioterrorism agents. However, currently available rapid testing has many limitations. Areas of improvement that need to be addressed include the ability to test for all or most know agents, improved collection methods, improved integration of diagnosis with use of medications and clinical care, use of diagnostics for epidemiology and vaccine development, increased sensitivity in adults and immunocompromised patients, and improved ease and accuracy of testing.

Rapid testing is currently available for RSV and influenza on a wide basis. There are significant limitations of these tests including sensitivities and specificities that are dependent on the quality of the sample collected, the timing of the illness, the intensity of the epidemic, and the population tested. These assays have been developed to allow for sentinel surveillance, although their positive predictive values are limited at the beginning and end of epidemics (see below). In addition, rapid testing helps to identify patients who may benefit from antiviral agents and to decrease the use of antibiotics and shorten the duration of hospitalizations. The available rapid tests for RSV and their respective characteristics have been reviewed elsewhere recently (Caliendo, 2002). Rapid testing for influenza uses three major methods: fluorescent antibodies (FA) and other antigen detection techniques, neuraminidase detection, and polymerase chain reaction (PCR). FA testing is rapid with a turn-around-time (TAT) of 20 min to 2 h. Unfortunately, it requires intact cells in the collected fluid, an expert reader, and a fluorescent microscope. It is able to distinguish between influenza A and B and has a sensitivity of 92–99% and a specificity of 96% (Hornsleth and Jankowski, 1990; Storch, 2003). It costs about US$ 2.50–7 to screen and US$ 8–18 to identify influenza. PCR takes slightly longer with a TAT of 6–12 h and requires an expert technician and specialized, expensive equipment. Unfortunately, PCR is not available at most centers as a clinical assay.

Very rapid tests include Becton–Dickinson Influenza A and Influenza A/B, enzyme immunoassays (EIA), Zstat Flu, an endogenous viral encoded assay (EVEA), FluOIA, an optical immunoassay (OIA), Quickvue Influenza, a lateral flow immunoassay (LFIA), and Binax Now Flu A and Flu B, an immunochromatographic membrane (ICT) assay. The ZStat Flu, QuickVue Influenza, and Binax Now FluA and FluB are currently CLIA-waived which allows for in-clinic testing. The features of the different assays have been summarized recently (Nicholson et al., 2003). Unfortunately, reported sensitivities and specificities of these various tests cannot be directly compared, since different ‘gold standards’, source populations, sample types (fresh versus frozen; nasal versus throat), and operators (i.e. research laboratory versus clinical laboratory versus physician office) were used to evaluate each test. Use of a particular rapid diagnostic test for influenza should be determined according to the level of identification needed (influenza A or B versus influenza A and B), price, CLIA status, specimen type, staff familiarity with methods, turn-around-time, and relative sensitivity and specificity of the test. Additionally, the predictive value depends on the prevalence of disease. For example, if the sensitivity and specificity of the test was 80%, and the prevalence of influenza was 5%, the positive predictive value (PPV) of the test would be 17%; if the prevalence was 20%, the PPV would be 50%. Rapid testing is best used in individuals with a strong pre-test probability of influenza during the influenza season. When clinically important (for example, in hospitalized patients), negative rapid test results should be confirmed by culture.

6. Pathogenesis and immunology

6.1. Immune responses to influenza and respiratory syncytial virus infections: lessons from knockouts (Tom Braciale, University of Virginia, Charlottesville, VA)

The response to respiratory viral infections is mediated through interactions within the mucosal surface which contains epithelial cells, alveolar cells, dendritic cells, and macrophages, the innate immune system, including natural killer cells, neutrophils, and macrophages, and the adaptive immune system, including B and T cells. These interactions occur as the result of mediators including preformed ones like the collectins, including surfactant protein A (SP-A) and D (SP-D), and inducible mediators, such as collectins, cytokines, chemokines, granule products, and antibodies. Several signaling pathways are used to modulate the immune response following infection. Viral infection of epithelium, interaction with pattern recognition receptors, such as toll-like receptors, and creation of Virus–collectin complexes results in upregulation of mediator genes, predominately cytokines and chemokines. Virus–collectin complexes also enhance phagocytosis. Viral proteins binding to B cell receptors results in increase antibody and

mediator production, while viral protein binding to T cell receptors results in activation of T cells and increased mediator production. The chemokines and cytokines that are elaborated in response to the infection then interact with receptors that result in chemotaxis and regulation of gene expression that ultimately results in the immune response.

To evaluate the role of specific mediators, receptors, or signaling pathways in response to respiratory virus infections, animal experiments, particularly with mice, are frequently used. Three types of mice can be used for such experiments: transgenic mice, where the gene encoding the molecule of interest is over-expressed or expressed in an abnormal location (tissue), knock-out mice, which have specific genes or gene segments deleted, and knock-in mice, which have mutated forms of the gene replacing the wild-type version. For example, collectin-deficient mice have been used in influenza and RSV infection models. SP-D deficient mice have increased sensitivity to RSV and influenza A infection, increased inflammation, increased lung virus titers with delayed virus clearance. SP-A deficient mice have a similar phenotype. These studies suggest that collectins regulate the innate and adaptive immune response.

Since the RSV-F protein binds to toll-like receptor 4 (TLR-4), TLR-4 deficient mice have been used in immunopathogenesis studies on the role of toll receptors in RSV infection. TLR-4 deficient mice had impaired clearance of RSV from lungs, but TLR-4 deficiency does not have an effect on influenza A clearance. TLR-4 deficient mice also had diminished pulmonary IL-12 synthesis after RSV infection and defective recruitment and activation of NK cells. TLR-4 is therefore considered a major mediator of RSV clearance (Haynes et al., 2001).

Interferons play a key role in control of many viral infections. Typically interferon receptor genes are knocked out instead of the interferon genes because numerous genes code for each type of interferon. Survival, lung virus titers, and clearance of influenza in IFN-α/β/γ receptor knock-out mice are not different from that observed in normal mice (Price et al., 2000). Interferon may limit influenza virus dissemination for those strains that can replicate outside of the respiratory tract, a process that is more difficult to model in mice.

Several knock-out mice have been used to better understand the impact of the adaptive immune response on influenza virus infection. B-cell function has been tested using μMT, J_H, and C_K knockouts, while CD8 and MHC class II knockouts have been used to understand the role of CD4 and CD8α and β2M knockouts have been used to understand the role of CD8 T cell. From studies with these knockouts, deficit in any component of the adaptive immune system results in enhanced susceptibility to infection, delayed virus clearance, and increased pulmonary inflammation and injury following influenza infection. Recovery from infection can be mediated by the remaining adaptive immune response in conjunction with the ongoing innate immune response. Experiments with these mice suggest that resistance to repeat infection and hetero-subtypic immunity is mediated in part by B cells.

The role of secretory antibodies has been studied using IgA, J chain, and poly-Ig receptor knockouts. These studies have demonstrated that secretory IgA is not required for recovery from pulmonary infection but that secretory IgA may enhance clearance of homotypic and heterosubtypic virus from the upper respiratory tract following infection.

Finally, the role of CD8 T cell lytic machinery has been studied using perforin, Fas, and Fas ligand knockout mice. Perforin deficiency has no impact on influenza clearance but does result in delayed clearance of virus and increased inflammation in RSV infection. Fas ligand deficiency has no impact on influenza clearance, but combined defects of perforin and Fas ligand results in delayed clearance of influenza by CD8 T cells.

Taken together, our understanding of the contributions of individual components of the immune system to the clearance of virus and survival of the host following influenza and RSV infection has been advanced by the use of genetically modified animals. Both the innate and adaptive host immune responses contribute to virus clearance and recovery from experimental influenza and RSV infection. Innate and adaptive host responses are cooperative and redundant. Immunologic memory in the respiratory tract may not be accurately reflected systemically. With greater understanding of the innate and adaptive immune response, it may be possible to develop vaccines with improved efficacy or with the ability to modulate the pulmonary injury.

6.2. Host genes associated with susceptibility to RSV (Jeremy Hull, John Radcliffe Hospital, Oxford, UK)

RSV is highly infectious and nearly all children have serological evidence of infection by the end of their second year. It is not known why some infected infants develop severe disease, whereas the majority have mild symptoms. In response to infection, the host produces a complex mixture of cytokines and other inflammatory mediators with anti-viral but also potentially pathological properties. Various clinical and experimental observations indicate that certain patterns of host response are associated with severe disease, but it has been extremely difficult to prove that these are causal determinants, rather than just correlates, of clinical outcome. To better understand the association between clinical response to infection (phenotype) and specific genes, genetic association studies need to be done. Genetic association studies can be done using a genome approach or using candidate genes. It has been estimated that genomic approaches would require study of at least 200,000 markers in several thousand subjects, a project which would cost many millions of dollars. For this reason candidate gene approaches are used by most groups.

Several candidate genes have been identified that may play a role in the immune response to RSV, including IL-5,

8, 10, and 13, CCR5, ICAM-1, IL-4 receptor, IFN-γ and IFN-γ receptor. The primary objective of current genetic association studies is to identify host factors that contribute to the development of severe lower respiratory tract disease following infection by RSV. These studies will address this problem through a molecular genetic approach, by investigating whether disease susceptibility is related to common polymorphisms in host genes that are thought to be critical for protection and/or pathogenesis. The UK study is enrolling infants, less than a year old, with severe RSV bronchiolitis. DNA samples from the patient, the patient's parents, and ethnically matched cord blood controls are collected and analyzed. To date, 700 cases have been collected, including 550 complete family samplings.

The first gene to be analyzed was IL-8, a potent neutrophil chemo-attractant and activator. We have previously demonstrated that IL-8 promoter polymorphisms may influence the level of gene expression. IL8-251A is associated with severe bronchiolitis. The odds ratio (OR) for bronchiolitis for a T to A change is 1.25 while AA to any other change is 1.47. There is also a suggestion that the IL8-251 polymorphism predisposes to subsequent wheezing illness after bronchiolitis. In a follow-up study of children who had been admitted to hospital with RSV bronchiolitis, IL8-251AA was found more frequently in children who experienced subsequent wheezing (29%). The frequency in children without wheezing was not different from controls who had not had bronchiolitis (17% versus 20%). Our current work is directed at fine mapping of the IL8 bronchiolitis susceptibility effect; preliminary studies suggest there is also a link with a RAS effector gene (*RASSF6*) (OR 2.1) that is located just downstream from the IL-8 gene locus.

We have also investigated variants of CCR5. CCR5 is the major receptor for RANTES, MIP-1α, and MIP-1β and has several polymorphisms that are associated with levels of CCR5 on the cell surface. Certain polymorphisms of CCR5 have been associated with progression of HIV. We have found that a CCR5 promoter SNP (single nucleotide polymorphism; CCR5-2459) is associated with more severe bronchiolitis (OR 1.5; OR 1.8 without other risk factors). In combination, the studies on IL8 and CCR5 suggest that the innate immune response of the airway epithelial cells to infection with RSV appears to be important in determining disease severity.

Another area of interest in RSV pathogenesis relates to chromosome 5q31–33, which contains several markers that have been associated with atopy, IgE levels, and wheezing illness. Several Th2 cytokine genes map to this region, including IL-4 and IL-13. We have found that a common haplotype of IL-4, which 77% of humans carry, is associated with increased risk of severe RSV bronchiolitis (OR 1.5). In a Korean study of 105 children under 24 months old with RSV lower respiratory tract infections and 315 blood donors, a different haplotype at IL-4 was also found to be associated with more severe disease (OR 1.63) (Choi et al., 2002). These results suggest that there may be a functional variant at this locus (identified by different haplotypes in different populations) that affects disease severity. In addition, we have preliminary data that suggests that a rare haplotype of IL-13, found in only 5% of humans, also affects the risk of bronchiolitis after exposure to RSV (OR 2.0).

In conclusion, data from genetic association studies indicate that both innate and adaptive immune responses are important in determining the severity of RSV disease. Genetic association studies should shed light on the pathogenesis of severe RSV disease, as well as providing information that may allow targeting of therapies to infants at especially high risk. This approach could be extended to other common respiratory viral infections, such as those due to influenza and rhinovirus, to understand differences in disease expression.

6.3. Infection of primary human airway cultures (Peter Collins, NIAID, NIH, Bethesda, MD)

Efficient RSV infection in HEp-2 and A549 cells involves binding to cell surface glycosaminoglycans (GAG) such as heparin sulfate and chondroitin sulfate. These GAGs are long chains of repeating disaccharides, typically 70–200 residues in length, and are present on surface proteoglycans of the extracellular matrix. HPIV3 attaches to sialic acid that decorates cell surface glycoproteins and glycolipids, although GAGs also have been reported to contribute to HPIV3 attachment. In contrast to the usual practice of employing monolayer cultures of immortalized cell lines, the present study investigated RSV and PIV3 infection of primary human airway epithelial (HAE) cells. These cells are grown on a support in culture and, upon the establishment of an air–liquid interface, differentiate into a multi-layered, pseudostratified, polarized tissue that closely resembles the airway epithelium in vivo with regard to morphology and functions, including mucus production and ciliary motion. The cilia are limited to the apical surface of the cells. Keratan sulfate is frequently present on ciliated cells and can detected immunologically.

Wild type recombinant RSV was genetically engineered to express the green fluorescent protein (rgRSV) from an added gene, so that infection could be monitored in real time in living cells. rgRSV could only infect the cells when applied to the apical surface and not the basolateral surface. Furthermore, the virus appeared to infect only ciliated, keratan sulfate-expressing cells. Mechanical damage to the tissue, which exposes underlying layers of cells, did not increase the range of infection, indicating that this reflected an authentic tropism rather than simple physical exclusion. Next, the virus was applied to cell cultures at different stages of development. Infection was very inefficient early in the development of the tissue (2 days after establishment of an air–liquid interface), but the efficiency increased with increasing differentiation, reaching a plateau after day 14. This corresponded with the appearance of ciliated cells. The virus was also shown to be shed from the apical surface at reasonably steady levels after infection ($\sim 10^7$ plaque forming units per ml of wash fluid). Timed microscopy demonstrated

well-defined spread of the RSV across the apical face consistent with cilia motion. Ribavirin and palivizumab inhibited infection of the cells. Thirty-six days after rgRSV infection, intact infected cells were readily detected but, interestingly, cytopathologic effect was minimal, although cells were noted to become rounder in the vertical dimension. These studies show that RSV infects only the apical surface and only keratan sulfate-containing ciliated cells. RSV infection has minimal cytopathic effect with an absence of syncytia, likely due to polarized expression of F protein.

A similar model was created using HPIV3 expressing GFP (PIV3GFP). Like RSV, HPIV3 appears to infect, bud, and spread via the apical surface only. HPIV3 also appeared to infect only ciliated cells, and appeared to infect keratin sulfate-containing cells, as well as a subset that lacked keratin sulfate. Unlike RSV, HPIV3 was able to infect undifferentiated cells as efficiently as differentiated cells. Infection appeared to be completely dependent on sialic acid on the apical cell surface. Lastly, HPIV3, like RSV, does not cause visible damage to the tissue and HPIV3 does not cause the change in cell shape associated with RSV. These model systems may help identify cellular receptors for RSV and HPIV3 and should be useful in studying the epithelial cell response to these infections.

6.4. Virus–bacteria interaction (Robert Sherertz, Bowman-Gray School of Medicine, Winston-Salem, NC)

Staphylococcus aureus is the most common cause of nosocomial infections in the United States. Nosocomial *S. aureus* outbreaks have been linked to healthcare workers such that concern has been raised that *S. aureus* could be spread through the air. Increased risk of *S. aureus* airborne transmission has been associated with increased *S. aureus* in the nose, male sex, snorting or sneezing, antibiotic use, cutaneous diseases, and viral infections. A phenomenon entitled the "cloud baby" was originally described in 1960 by Eichenwald in which newborn infants nasally colonized with *S. aureus* had airborne dispersal of *S. aureus* associated with an upper respiratory tract viral infection (URI) (Eichenwald et al., 1960). Cloud Babies were capable of causing outbreaks both in the hospital and at home. The concept was expanded to include adults when an outbreak of *S. aureus* infections occurred in two newborn nurseries associated with a single nurse. During the outbreak investigation, it was noted that the likelihood of this nurse transmitting *S. aureus* increased five-fold during periods of time when she had an upper respiratory infection (Belani et al., 1986).

The possibility that "cloud adults" exist was substantiated during an investigation of another point-source outbreak of *S. aureus* nosocomial pneumonias. This outbreak was tied to a single physician who had a URI. The outbreak lasted 3 weeks which was coincident with his respiratory symptoms. This physician was then experimentally infected with rhinovirus and open agar plates were placed around him to detect airborne dispersal. Following rhinovirus infection, there was a 40-fold increase in airborne dispersal of *S. aureus* and this increase could be decreased 75% by wearing a mask (Sherertz et al., 1996).

To further investigate the concept of a "cloud adult", a special chamber was designed in which laminar airflow was maintained and HEPA filters were used to purify the air. Airborne dispersal of *S. aureus* was found to be greatest when street clothes were worn into the chamber, less when sterile garb was donned without a mask, and least when the sterile garb with mask were used. The greatest amount of *S. aureus* was cultured after the volunteers were infected with rhinovirus. There was an approximately two-fold mean increase in airborne dispersal of *S. aureus* following experimental rhinovirus infection with peak airborne dispersal as high as 100-fold greater than baseline. Masks did not blunt this effect but wearing barrier garb (gown, gloves, booties, cap) did, suggesting that in most volunteers the airborne dispersal is not coming in real time directly from the nose. Independent predictors of detecting *S. aureus* in the area around the volunteers included wearing street clothes, sneezing, and rhinovirus infection. Cough and the severity of the cold were not related to *S. aureus* colony counts, and the greatest colony counts were obtained after the catarrhal phase of the illness.

Recent studies have shown that coagulase-negative *Staphylococci* have increased airborne dispersal after a rhinovirus infection suggesting that the "cloud phenomenon" may occur with other organisms. This finding may be quite important as it suggests a possible mechanism for the outbreaks of meningococcal, pneumococcal, and streptococcal (Group A) infection that occur in the community and in semi-closed populations as an aftermath to outbreaks of influenza and viral URI.

One potential hypothesis to explain the pathogenesis of the "cloud adult" is that infection results in inflammation that in turn reduces the diameter of the nasal passage and increases the rate of airflow and associated aerosolization of particulates, including bacteria, by the venturi effect. To test this hypothesis, volunteers with allergic rhinitis who had their medication held but did not have culturable virus were placed into the test chamber. After stopping their medications, there was increased airborne dispersal of *S. aureus*. The inflammatory response in the nose, not the rhinovirus itself, may be central to the mechanism of airborne dispersal.

6.5. Regulation of epithelial cell IL-5 production by RSV and IL-4 (Mihnea Zdrenghea, Imperial College, London, UK)

RSV bronchiolitis in infancy is strongly associated with the later development of asthma (Stein et al., 1999) and RSV is also implicated in virus induced asthma exacerbations. Protective immunity to re-infection with RSV is incomplete and of short duration; previously infected infants and adults remain susceptible to re-infection with antigenically closely related viruses or the identical virus strain. Recent evidence

suggests that virus-induced asthma exacerbations may be associated with deficient type 1 immunity (Papadopoulos et al., 2002d). A deficit in IFN-γ/IL-4 mRNA ratio and IL-18 mRNA expression by peripheral blood mononuclear cells (PBMC) has been observed in infants that developed RSV bronchiolitis compared to infants from the same birth cohort with a normal response to RSV infection (Legg et al., 2003).

IL-15 is a type 1 inducing cytokine produced by resident airway cells in response to inflammatory stimuli such as infection. By promoting (i) long-term survival of NK and memory $CD8^+$ T cells and (ii) type 1 function (IFN-α production and cytotoxicity) in NK, NK T cells and memory $CD8^+$ T cells, IL-15 plays an important role in antiviral immune response (Biron et al., 1999; Carson et al., 1997; Kennedy et al., 2000; Liu et al., 2002; Zhang et al., 1998).

We hypothesized that in a type 2 cytokine rich environment (as occurs in asthmatic airways), virus-induced production of type 1 cytokines is down-regulated. To test this hypothesis, we studied RSV-induced production of IL-15 in respiratory epithelial cells in the absence and presence of IL-4. A549 cells, a human lung type 2 alveolar carcinoma cell line, were infected with RSV A2 strain in the presence of 10 ng/ml of IL-4 or in its absence (control). RSV induced IL-15 production in A549 cells in a time- and dose-dependent manner. Experiments conducted in the presence of IL-4 showed a down-regulation in IL-15 production by RSV infection.

Decreased production of IL-15 in the lung in respiratory viral infections in asthmatic subjects may be a possible pathway for deficient antiviral immune responses, delayed virus eradication and virus-induced exacerbations of atopic asthma. Further study directions include repeating experiments on other cell lines and on primary bronchial epithelial cells, gene expression studies to define the mechanisms of RSV-induced IL-15 production and its down-regulation by IL-4, and comparison of IL-15 production during respiratory viral infections in normal and asthmatic subjects.

7. Clinical

7.1. The role of RSV: childhood wheezing, 'asthma', and atopy (Peter Openshaw, Imperial College, London, UK)

Asthma, by definition, is reversible airflow obstruction (change in FEV_1/PERF >14%), with bronchial hyperresponsiveness to irritants such as exercise, chemicals, allergens, or cold air. Most patients have wheezing, cough, or shortness of breath, and all have evidence of increased inflammation in the lungs. Wheezing can be classified into four types, predominating at distinct ages: transient infant wheeze (likely related to exposure to smoke or small lungs); early wheeze (typically after a viral illness); classical atopic asthma (which tends to be familial) and chronic obstructive airway disease (typically resulting from chronic irritant exposure, such as smoke, throughout a person's lifetime). Of 826 children followed from birth until age 6, 51.5% never wheezed, 19.9% had transient wheezing, 15% had onset of wheezing after 3 years of age, and 13.7% had persistent wheezing (Martinez et al., 1995). Five to six million American children have asthma, making it the most common chronic disease in children. It is estimated that there are 470,000 hospitalizations per year, 5000 deaths per year, and an estimated excess expenditure of over US$ 6 billion per year worldwide as the result of asthma exacerbations.

As a result of this significant burden, many have studied asthma and its relationship to respiratory viral diseases, particularly RSV. In one study, 47 children hospitalized for RSV lower respiratory tract illness (LRI) during the first year of life were compared to 93 controls with no or mild RSV upper tract illness. Patients with lower tract illness had a significantly increased risk of wheezing by 7 years of age (Sigurs et al., 2000). Another study compared 207 children with mild RSV LRI to control children without LRI during the first 3 years of life. The patients that had LRI has statistically significant increased frequency of wheeze compared to the control through age 11 (Stein et al., 1999). In total, nine controlled studies with 2–13 years of follow-up have been conducted, and all demonstrated increased frequency of lower respiratory tract symptoms and/or airflow obstruction after bronchiolitis; of all enrolled patients in these studies, 82% has lower respiratory symptoms within 2 years, 69% within 3.5 years, 55% within 4–5 years, and 31% within 6–8 years (Hall et al., 1984; Henry et al., 1983; Webb et al., 1985).

To further investigate a potential link between RSV bronchiolitis and asthma, Wenzel and colleagues conducted a long-term follow-up study of 13 children with bronchopulmonary dysplasia (PBD) who were treated with RSV polyclonal immunoglobulin (RSV-Ig) during infancy and 26 age-matched controls with BPD who did not receive RSV-Ig. Unfortunately, the groups were not matched for sex (more male children in the treated group), for RSV LRI (6/13 versus 21/26), or for cat exposure. However, 7–10 years after enrollment, RSV-Ig treated children had significantly better FEV1/FVC ratios and airway conductance, in addition to less atopy, missed school, colds, asthma attacks, hospitalizations, and use of asthma medications (Wenzel et al., 2002).

These results raised the question of whether normal lungs become asthmatic after viral infection or if weakened lungs that would become asthmatic without infection are also at increased risk of wheezing after viral infection. To address this question, we collected peripheral blood mononuclear cells from 37 7–8-year-old children with a history of RSV bronchiolitis and 70 without a history of RSV bronchiolitis. These PBMCs were thawed, stimulated with a number of antigens, and then had IL-4 and IFN-γ ELISPOT and proliferation assays performed. Bronchiolitic patients had increased IL-4 response to RSV and Fel d antigens, but not to other tested antigens (Der p, Bet v, tetanus toxoid, PHA, and media) (Pala et al., 2002). A second study took mice and exposed them to intranasal ovalbumin followed

by intravenous ovalbumin 18 days after initial exposure. Mice who were exposed to intranasal ovalbumin after being exposed to RSV or influenza A had an anaphylactic reaction to intravenous ovalbumin, while mice exposed without infections had minimal symptoms (O'Donnell and Openshaw, 1998). Taken together, these results suggest that mild infection results in low level IFN-γ production, while bronchiolitis results in increased IL-4 production in addition (Pala et al., 2002).

The age of acquisition of RSV infection may impact on future asthmatic sequelae. Most cases of RSV bronchiolitis occur prior to 4 months of age. At the time of birth, neonates have a strong Th2 immune response, whereas Th1 response matures with exposure to environmental antigens. Viral and gastrointestinal infections promote Th1 responses, which are responsible for protection against atopy. Such immune maturation hypotheses have been documented by stimulating tonsil extracts from children with RSV and PHA in vitro; these stimulations result in increasing levels of IFN-γ with increasing age. When mice are infected with RSV at 1 day, 1, 4, and 8 weeks of age and then reinfected at 12 weeks of age, infection of older mice resulted in increased viral load, likely secondary to larger lungs with more cells to infect, but similar rates of clearance of virus. Secondary infection caused substantial weight loss in mice primarily infected at 1 day and 1 week, but not in mice initially infected at 4 or 8 weeks. Mice primarily infected at 1 day and 1 week had increased number of total cells, CD8 T cells, eosinophils, and neutrophils extracted from the lungs. Mice infected at 2 and 4 weeks had increased production of IFN-γ and decreased production of IL-4 compared to mice infected at 1 day and 1 week (Culley et al., 2002). This data suggests that the nature of the secondary response is determined by the age at primary infection. RSV infection early in life sensitizes for severe response to viral challenge later in life and T-cell priming in the neonatal period may explain this association. Delaying RSV infection beyond infancy could have long-term benefits in terms of less severe disease, decreased atopy, and lower frequencies of asthma. Potential mechanisms for this delayed effect include chronic, persistent, or latent viral infection, remodeling of the lung after infection, permanent epithelial damage, immunologic priming or tolerance, or bystander antigenic sensitization. Further studies will need to be done to investigate these alternatives.

7.2. The allergy–virus interaction: good in the long-term and bad in the short-term? (Sebastian Johnston, Imperial College London, UK)

Several studies suggest that some infections in infancy protect against asthma and allergic disease while lower respiratory tract viral illness are associated with wheezing later in life. In general infections induce type 1 responses (IFN-α, IL-12, IL-15, IL-18, IFN-γ) while allergic disorders are associated with type 2 responses (IL-4, IL-5, IL-9, and IL-13); type 1 and type 2 responses are mutually counter-regulatory. Immune responses at birth are skewed toward type 2, and microbial exposure is required to develop type 1 responses, so environmental exposure early in life likely contributes to maturation of the immune system. Children from large families and those who are lower in birth order, both markers for increased exposure to microbes, are at reduced risk of developing hay fever, eczema, and allergen sensitization. Likewise, hepatitis A, likely a marker of load of infectious exposure, is associated with decreased risk of allergies (Matricardi et al., 2002).

Respiratory virus infections have been associated with an increased risk of wheezing illness and atopic sensitization. RSV has the strongest association (Sigurs et al., 2000) but the Tucson Children's Respiratory Infection Study has shed light onto other infections. Children were initially followed until 3 years of age and had samples collected for virologic studies when the child experienced any signs or symptoms of lower respiratory tract illness. Children then had follow up visits at 6, 8, 11, and 13 years of age to evaluate for wheeze. There was increased risk of later wheezing when lower respiratory tract illness had developed as the result of RSV infection. However, lower respiratory tract illnesses resulting from other virus types were also significantly associated with increased risk of wheezing later in life (Stein et al., 1999). Another study found substantial contributions of rhinoviruses to the severity of bronchiolitis (Papadopoulos et al., 2002c). Potential explanations for the relationship of early virus-related lower respiratory illness including RSV bronchiolitis is that patients with lower respiratory illness/bronchiolitis may have smaller airways, deficient type 1 or increased type 2 immunity, or develop increased allergen sensitization during acute infection.

To evaluate these potential explanations, Legg and associates collected nasal lavages on days 1, 2 and 5–7 of illness and peripheral blood mononuclear cells on days 5–7 of illness from children in their first year of life with proven RSV infection. At both time points, infants with bronchiolitis had a higher IL4/IFN-γ and IL-10/IL-12 ratios in nasal lavages and a depressed level of the IFN-γ/IL-4 mRNA ratio and of IL-18 mRNA expression in blood mononuclear cells compared to infants with URI. Slower clearance of virus (PCR viral load) was seen in patients with bronchiolitis (Legg et al., 2003). From these results, the authors concluded that RSV bronchiolitis is associated with deficient type 1 immunity and impaired virus clearance (Mallia and Johnston, 2002).

Day care attendance and having greater than two siblings increases the risk of wheeze early in life but decreases risk later in life (Ball et al., 2000). Others have also found differences in risk of wheezing between patients with URIs compared to those with LRIs. Patients with two or more episodes of URI before 1 year of age had a 50% reduced risk of asthma at 7 years of age, while patients who had LRI with wheeze were at increased risk (Illi et al., 2001).

In conclusion, it appears that the risk of allergic disease later in life can be reduced by high overall load of infectious

disease (including respiratory viral infections) early in life, but exposure to respiratory infections early in life causes increased morbidity at that time. Likewise, individual infections in subjects at risk, such as those with deficient type 1 immunity or small airways, result in infections that are more severe, more likely to involve the lower respiratory tract, and are strongly associated with wheezing later in life.

7.3. Respiratory virus infections in transplant recipients (Michael Boeckh, Fred Hutchinson Cancer Research Center, Seattle, WA)

Increasingly, respiratory viruses are being recognized as significant pathogens in transplant recipients. Serious illness due to respiratory viruses appears most common in hematopoietic stem cell transplant (HSCT) recipients and lung transplant recipients. The most frequently isolated respiratory viruses are respiratory syncytial virus, parainfluenza, influenza viruses, and rhinoviruses. A targeted surveillance system has found an attack rate among the approximately 4800 patients who received HSCT at the Fred Hutchinson Cancer Research Center (FHCRC) over the past 12 years to be 7% for PIV, 4% for RSV, and 1% for influenza. Most respiratory virus infections (RVIs) in the Seattle area, like most temperate areas, occur during the winter months (November to May in Seattle). Our retrospective review found that respiratory viral pneumonia was associated with an increased risk of death (HR 2.2 for RSV, 3.4 for PIV, and 2.4 for influenza). Likewise, patients with respiratory viral infections were at increased risk for invasive aspergillosis (HR 2.1) (Marr et al., 2002). This finding suggests that the clinical effects of RVIs are likely both from direct lytic effects and indirect immunomodulatory effects.

Recently, the group at the FHCRC has conducted retrospective studies of >4000 HSCT recipients to determine the incidence, risk factors, and outcomes of respiratory viral illnesses. Risk factors for acquisition of RSV, PIV, and influenza were analyzed. Season, male sex, and transplant type were associated with increased risk of developing RSV infection; unrelated donor status was associated with PIV infection; and season, female sex, and underlying malignancy were associated with increased risk of influenza infection. Data from other studies suggest that CD4 lymphopenia also enhances the risk of acquisition of a respiratory viral illness in a dose-dependent fashion. Lymphopenia is also associated with increased risk of progression to lower respiratory tract disease. Steroids were noted to be protective against progression in influenza infection but to be a risk factor, in a dose-dependent manner, for progression in PIV infections (Chakrabarti et al., 2001; Ljungman et al., 2001; Nichols et al., 2001).

HSCT recipients with RVIs typically develop upper respiratory tract infection initially. A smaller percentage progress to lower respiratory tract involvement after a median of 7 days. Unfortunately, it is not yet possible to determine accurately which patients will progress to lower tract disease. RSV has been recognized to have one of the highest rates of progression to the lower respiratory tract. Most patients develop RSV during the first 3 months post-transplant with a median of onset ranging 40–50 days post-transplant (Falsey and Walsh, 2000). Most patients typically presented with cough (87–100%), fever (65–100%), rhinorrhea or sinus congestion (61–82%) and less frequently with sore throat (11–27%) and otalgia (36%). A large percentage of patients have rales or rhonchi on exam (50–100%), have chest X-ray infiltrates (58–75%), and manifest wheezing (35–56%) or dyspnea (36–45%).

Once RSV has progressed to pneumonia, there is no clear prospective data to direct management. Several, mostly small, retrospective studies have documented poor response to intravenous ribavirin alone (20–100% survival) or in combination with aerosolized ribavirin with (50% survival) or without (40% survival) IVIG (Lewinsohn et al., 1996; Ljungman et al., 2001). Better results have been documented with aerosolized ribavirin alone (22–66% survival) (Harrington et al., 1992; Ljungman et al., 2001), or in combination with IVIG (50–68% survival) (Ghosh et al., 2001; Ljungman et al., 2001; Whimbey et al., 1995), RSV-Ig (86–91% survival) (DeVincenzo et al., 2000; Small et al., 2002) or palivizumab (83%) (Boeckh et al., 2001). These data suggest that optimal therapy for RSV pneumonia involves the combination of aerosolized ribavirin plus an antibody preparation. Starting antiviral therapy early impacts survival. If combination therapy is started before respiratory failure begins, 78–80% of patients survive, compared to none who have therapy begun after respiratory failure. Potential risk factors for increased mortality in patients with RSV pneumonia includes the co-pathogens, concomitant immunosuppression (steroids, anti-lymphocyte antibodies), lymphopenia, preexisting lung disease, and the therapy used, including the time of onset. Prevention strategies should be explored to limit the frequency of RSV disease. Infection control is an important component to prevent infections, but is of limited value when the patient leaves the hospital or clinic. Use of RSV-Ig or palivizumab for prophylaxis might be beneficial, although the two preparations have never been studied in this population in a randomized fashion and would be very expensive to use. Preemptive therapy, with aerosolized ribavirin, could be used in patients with asymptomatic shedding or RSV URIs, although early detection methods limit the ability to implement preemptive management of these infections. Additionally, these preemptive strategies have not been tested for RSV in randomized trials.

As a result of the potentially devastating outcome of RSV and other RVIs, there has been some concern about the need to delay HSCT in symptomatic patients. In a retrospective case series of 10 patients who underwent autologous HSCT for advanced multiple myeloma who also had active RSV URI, none of the patients progressed to LRI, required transfer to the ICU, or died after transplantation as the result of RSV (Aslan et al., 1999). One patient developed tracheobronchitis and required oxygen transiently. The strategy

currently used is to delay transplant if upper respiratory symptoms are present and not do the transplant until cessation of symptoms and the nasal wash is negative for virus (CDC, 2000). At FHCRC we found that 37 patients who developed RSV URI prior to transplant. Thirty-one had the URI before conditioning and two developed pneumonia after transplantation with response to treatment. Six were discovered after starting conditioning. Of the three that had the transplant aborted, none developed RSV pneumonia, whereas two of those that continued with transplantation developed pneumonia and one died. This same study documented pre-transplant shedding in patients for up to 42 days with most patients having shedding of virus for at least 1 month. Delay in transplantation did not result in progression of the underlying disease. Thus, delay of transplant for at least 1 month should be advised for all myeloablative transplants (CDC, 2000).

7.4. Respiratory syncytial virus infection in patients with hematological malignancies: results of a prospective study (Nuria Rabella, Barcelona, Spain)

The current study was designed to assess the clinical and microbiological characteristics of RSV infection in patients with hematological malignancies. Adult in- and out-patients with hematologic malignancy, including HSCT recipients, were included in the study if they had signs and symptoms of an upper or lower respiratory tract infection. A total of 251 nasopharyngeal aspirates and 19 bronchoalveolar lavages were collected during 256 episodes of respiratory infection in 198 patients from October 1999 to June 2002. One or multiple respiratory viruses, were identified in 122 episodes (48%). Influenza A virus was found in 67 cases, followed by respiratory syncytial virus in 24, enteroviruses in 12, adenoviruses in 12, influenza B virus in 8, parainfluenza viruses in 10 (5 PIV 1 and 5 PIV 3) and two rhinoviruses. In eight cases, a mixed viral infection was diagnosed.

Among the 24 episodes of RSV infection, RSV was detected alone in 18, while eight patients also had infections with influenza A and in one with CMV. Most (88%) of the patients cleared the infection during the first 2 weeks, while one patient each had detectable virus until day 18 and week 5. Thirty-eight percent of the patients acquired their RSV infection in the hospital after a median of 15 days (range: 5–26 days). Upper respiratory tract infection was diagnosed in 17 patients, LRI in 7, and only one patient with upper respiratory tract infection at presentation progressed to LRI. Six patients received inhaled ribavirin and only one of the treated patients died. This study prospectively confirmed that RSV occurs typically in the winter months, can be nosocomially transmitted, and can frequently progress to involve the lower airways. Most patients clear the infection during the first 2 weeks after infection. RSV infection was associated with a low mortality in this heterogeneous population, despite 33% having lower respiratory tract symptoms.

7.5. Viral etiology of acute expiratory wheezing in children (Tuomas Jartti, University of Turku, Turku, Finland)

Up to 20–25% of all children have at least one episode of wheezing, and most cases of asthma exacerbations are the result of viral respiratory infections (Glezen et al., 2000; Habbick et al., 1999; Hesselmar et al., 2000; Johnston et al., 1995; Martinez et al., 1995; McIntosh, 1976; Mertsola et al., 1991; Rakes et al., 1999). A prospective study was conducted to determine which respiratory viruses are isolated from children in Finland who are hospitalized for acute expiratory wheezing. A total of 293 hospitalized children (median age: 1.6 years, range: 3 months to 15 years) were recruited from September 2000 until May 2002 in Turku, Finland. Patients with chronic lung disease, heart disease, and chronic steroid use were excluded from the study. In addition to serologies, nasal aspirates were tested by PCR for rhino-, entero-, corona- and metapneumo-virus (hMPV), antigen detection and culture for influenza A and B, parainfluenza type 1, 2 and 3, adenovirus and RSV, and culture for rhino- and enterovirus. A potential causative viral agent was found in 88.7% of cases: RSV 27%, entero- 25%, rhino- 22%, nontypable picorna- 16.4%, adenovirus 5%, hMPV 4%, parainfluenza type 1/3 5%, influenza A and B both 1% and coronavirus 1%. Eighteen percent of patients had multiple viruses detected. Patients with rhino- and entroviruses were slightly older (median: 2.2 years) than patients with RSV and hMPV infection (median: 0.9 years). Patients with RSV and hMPV typically had longer durations of hospitalization than patients with other viral pathogens detected. RVIs were associated with almost all episodes of wheezing leading to hospitalization in this study. This study was also one of the first to demonstrate the contribution of hMPV, particularly during winter epidemics, and its correlation with wheezing (Jartti et al., 2002b; van den Hoogen et al., 2001). The prevalence of enterovirus was higher in this study than previously reported, and the results confirm the large contribution of picornaviruses, particularly rhinoviruses, to wheezing episodes in children.

8. Vaccinology

8.1. Live attenuated pandemic influenza vaccines (Kanta Subbarao, NIAID, NIH, Bethesda, MD)

One of the many key components to pandemic preparedness is the development of pandemic vaccines before the pandemic arrives. Live-attenuated influenza vaccines (LAIV) are particularly appealing as they are typically easier to deliver than injectable vaccines. Unfortunately, there are several issues that currently limit the development of these vaccines including the need to generate candidate vaccines in a BSL3+ facility, the wide range of avian viruses, the choice of vaccine development strategy (reverse genetics or reassortment), choice of donor virus (PR8 or LAIV), and the need to do extensive safety and immunogenicity testing

of candidate in animals and humans. An additional issue is selection of the optimal serologic assay (HAI, Nt, SRH) for determining immunogenicity and defining an appropriately protective titer.

The avian influenza H5N1, H9N2, H5N2, H7, and H6 subtypes have recently been identified as high priority for pandemic vaccine development. Three candidate vaccines against H5N1, H5N2, and H9N2 are in early states of development. The H5N1 × AA candidate vaccine is a modified HK H5N1 × A/AnnArbor/6/60 cold-adapted, 6–2 reassortant virus that has been developed by reverse genetics as part of a collaboration between Aviron and CDC. The virus replicates to 8.5–9.4 $\log_{10}$ in eggs and has a mutagenized HA that decreases its cleavability by endogenous proteases. The virus grows at 33 °C but not 39 °C and is not pathogenic in chickens or ferrets. Studies in chickens demonstrated that the two candidate vaccines, MVS156 and MVS483, are immunogenic and reduce mortality after challenge (Li et al., 1999).

The H9N2 G9/AA vaccine is a temperature-sensitive, cold-adapted, 6:2 reassortant virus generated by genetic reassortment in SPF eggs as part of a collaboration between NIH and CDC. In mouse studies, intranasal application of the G9 reassortant virus resulted in low titers of the virus in the nose and minimal virus in the lungs. The vaccine was also immunogenic in the mice and prevented challenge infection. Finally, an H5N2 × AA cold-adapted virus that was developed also through genetic reassortment as part of a collaboration between NIH and CDC has been created. Early studies of this candidate are on-going.

The first two vaccines are moving into human proof-of-principle trials. The trials will be conducted with isolated volunteers who are vaccinated during the summer when influenza activity is minimal in the United States. The key properties of the vaccine to be studied include infectivity, level of attenuation, and immunogenicity. It is hoped that these and future studies will allow the development of seed lots of vaccine viruses for a range of avian viruses. Additionally, alternative substrates for vaccine production and new adjuvants will need to be tested to enhance our armamentarium against pandemic influenza strains.

8.2. Live-attenuated, cold-adapted trivalent influenza vaccine (John Tam, The Chinese University of Hong Kong, Hong Kong SAR, China)

The trivalent cold-adapted influenza vaccine (CAIV-T) we tested from Wyeth Laboratories has a major advantage of being stable in liquid form without the need for freezing. The study (D153P501) was designed to determine the efficacy of CAIV-T in a diverse Asian population of children age 12–36 months against culture-confirmed influenza illness, to assess the efficacy over serial influenza seasons, and to investigate the impact of CAIV-T on acute otitis media. Subjects were blindly randomized in a 3:2 fashion to receive either the CAIV-T or placebo during the first year of the study. A booster dose was given 35 days after the initial dose and a third dose was given 1 year after the first. There were four study groups: CAIV-T (year 1, dose 1)/CAIV-T (year 1, dose 2)/CAIV-T (year 2); CAIV-T/CAIV-T/placebo; placebo/placebo/CAIV-T; placebo/placebo/placebo. The studied enrolled 3174 healthy children between the age of 12 and <36 months whose parent or legal guardian gave written consent over 5 weeks starting in September 2000.

During the first year of the study, a total of 1900 patients (46.2% female, mean age 23.5 months) received CAIV-T and 1274 (47.7% female, mean age 23.4 months) received placebo. Most of the patients were Chinese (34.8%), Thai (27.5%), or Filipino (25.9%). During the first year of the study, six different influenza virus strains were identified from 181 subjects; four were antigenically similar to the vaccine, but two (B/Hong Kong/22/2001-like and B/Hong Kong/330/2001-like) were not. As shown in Table 2, the efficacy of the vaccine against culture-confirmed illness was estimated to be 74.3% (95% CI 64.1–81.8) overall and the point estimates of efficacy against H1, H3 and B influenza infection were 82.3, 88.8, and 50, respectively.

From the interim analysis of the first year's data, it appears that CAIV-T confers substantial efficacy against culture-confirmed influenza in Asian children, despite regional differences in influenza circulation. Additional data about the impact on AOM and the results of the second year of the study are in progress.

8.3. Status of the FluMist intranasal influenza vaccine (Harry Greenberg, MedImmune Vaccines, Mt. View, CA)

[Editorial note: FluMist is an intranasal live-attenuated, cold-adapted, trivalent influenza vaccine that was approved by the FDA for use in healthy children and adults ages 5–49 years in June 2003.]

Approval of the CAIV-T vaccine was slow because of concerns about safety and efficacy in certain age groups. The vaccine has been shown to be genetically stable; viruses that have lost their temperature sensitivity or cold-adaptation

Table 2
Efficacy of CAIV-T on culture-confirmed influenza infection

	CAIV-T subject (%)	Placebo subject (%)	Efficacy (%) (95% CI)
Number of subjects	1656	1115	
All culture-confirmed influenza	50 (3.0)	131 (11.7)	74.3 (64.1, 81.8)
A/H1N1 (including H1N2)	20 (1.2)	76 (6.8)	82.3 (7.07, 89.8)
A/H3N2	4 (0.2)	24 (2.2)	88.8 (67.3, 97.2)
B	26 (1.6)	35 (3.1)	50.0 (14.5, 71.1)

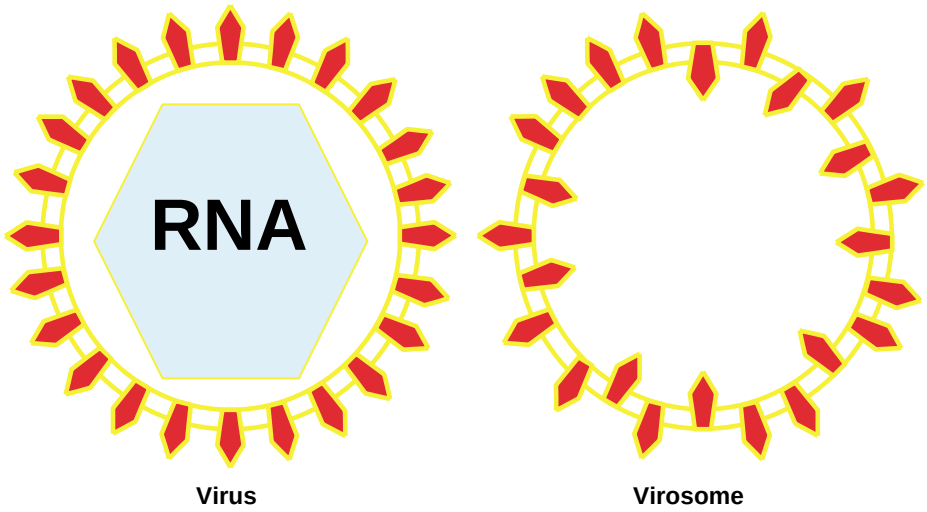

Fig. 1. Schematic of influenza virus and influenza virosome.

have not been recovered from vaccinated patients. Likewise, transmission of the vaccine virus is very infrequent; only one case has been documented in which a child in a day care setting acquired the vaccine strain from another vaccinated child. The child who inadvertently acquired the virus did not have symptoms distinct from other placebo recipients. The two major concerns on the part of the FDA after their initial advisory panel review in 2001 related to the questions of increased risk of pneumonia and asthma in vaccinated individuals. Subsequent analyses of the original clinical trials data failed to demonstrate an increased risk of pneumonia in vaccinated individuals. Data from a large study in the Northern California Kaiser system did demonstrate an increased number of medical encounters for asthma in vaccinated children compared to placebo vaccinated patients, but most of the visits were outpatient clinic visits for asthma management and did not temporally cluster within the 42-day post-vaccination observation period. Many of these subjects had a history of asthma at the time of enrollment and none required hospitalization. No asthma signal was detected in children above the age of 5 years. The FDA advisory committee felt that there was adequate data on the efficacy and safety of the vaccine in children >59 months of age and adults younger than 50 to recommend approval for this age group. As a result, further studies are needed in the age groups that have not been as completely studied, including young children and otherwise healthy adults aged 50–64 years.

8.4. The virosome concept for influenza vaccines (Jan Wilschut, University of Groningen, Groningen, The Netherlands)

Virosomes are functionally reconstituted, non-infectious viral envelopes retaining the cell entry properties of the native virus but lacking the RNA core. In effect, virosomes represent a functional reconstitution of the hemagglutinin (HA). One key difference between virus envelopes and the virosome is that the virosome has HA molecules extending bidirectionally on the external and internal surfaces of the membrane (Fig. 1). Virosomes are produced by first solubilizing inactivated influenza virus in an excess of the nonionic detergent octaethyleneglycon-*n*-dodecyl monoether (Stegmann et al., 1987).

There are several theoretical advantages of virosomes as vaccines. Virosomes maintain normal receptor binding and low-pH-dependent membrane fusion that occurs with intact influenza viruses. Antigens may also be encapsulated within the lumen of the virosome, so that delivery of the antigen occurs intracellularly and the immune system can react against both the antigen within and on the virosome. Theoretically, once taken up by an antigen presenting cell (APC), the virosome would fuse from within the acidic endosomal cell compartment, resulting in delivery of virosome-encapsulated antigen to the cell cytosol, while surface-associated antigen—including part of the HA—would remain behind in the endosome. In the cytosol, the antigen would be processed by proteasomes. Peptides produced would bind in the ER to MHC class I molecules to be ultimately presented on the APC surface, priming $CD8^+$ T cells to mature into cytotoxic T lymphocytes (CTLs). Within the endosome, virosomal antigen would be degraded to allow peptides to bind to MHC class II molecules and activate $CD4^+$ T helper cells (Fig. 2).

To document the ability of virosomes to activate both MHC class I and II mediated immune pathways, ovalbumin was included within virosomes. The ovalbumin-containing virosomes were then incubated along with cultured murine dendritic cells in the presence of T cells in vitro to assay for MHC class I or class II presentation using specific $CD8^+$ or $CD4^+$ T cells. The experiment was repeated with fusion-inactivated ovalbumin virosomes. Fusion-active ovalbumin virosomes, but not fusion-inactive ovalbumin virosomes, empty virosomes, or free ovalbumin, resulted in efficient antigen presentation via the MHC I pathway, while

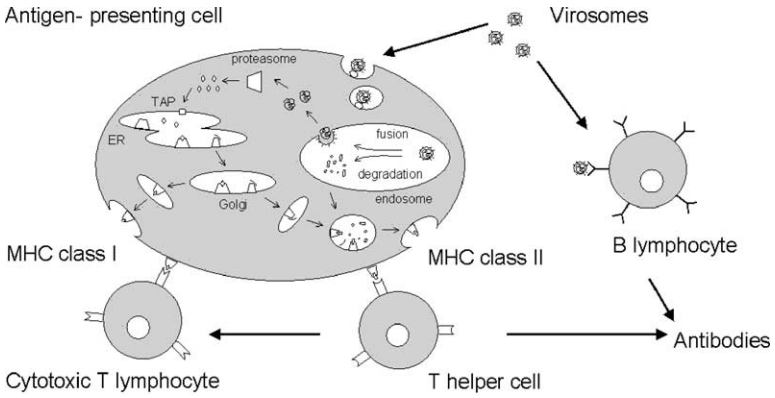

Fig. 2. Schematic of virosome interaction with the immune system.

both fusion-active and fusion-inactive ovalbumin virosomes accessed the MHC II pathway (Bungener et al., 2002b). Accordingly, in an in vivo mouse model, fusion-active ovalbumin-containing virosomes induced a powerful class I MHC-restricted CTL response against the ovalbumin antigen (Bungener et al., 2002a).

Despite a concern that pre-existing antibodies against HA would block virosome-mediated CTL priming, mice with a high pre-existing antibody titer against virosomal HA developed a strong ovalbumin-specific CTL response after subsequent immunization with ovalbumin-containing virosomes. It appears that antibody-opsonized virosomes are actively processed by dendritic cells through uptake via Fc receptors.

In conclusion, virosomes deliver antigens to dendritic cells for presentation on MHC class I and II molecules. Fusion activity of the virosomes is essential for MHC class I presentation. Virosomes induce maturation of dendritic cells as evidenced by upregulation of maturation markers including MHC class I and II molecules. Although virosomes induce a mixed Th1/Th2 response, the response is skewed toward Th1 supporting priming of CTL activity. As a result of their broad activation of the immune system and their ability to carry antigens, virosomes hold significant promise for vaccine development. Virosomes could also be used as a platform for inclusion of immunomodulators or adjuvants to be used as potential intranasal and/or pandemic influenza vaccines. Newer influenza virosome vaccines could be supplemented with other influenza antigens, such as the NP, M1, or M2, for further induction of humoral and CTL responses. Potential other applications include vaccine development for bioterrorism agents and therapeutic cancer vaccines.

It is hoped that a virosomal influenza vaccine, initially developed by Berna Biotech and now under development by Solvay Pharmaceuticals, will be clinically available during the 2003–2004 influenza season in The Netherlands and Switzerland. Fifteen clinical studies with 2970 patients have been done on the vaccine, called Influvac®. The vaccine has been well tolerated and is at least as effective as conventional subunit vaccines. The vaccine has resulted in seroprotection in 77–100 and 60–100% and seroconversion in 52–87 and 30–77% for patients aged 18–60 and >60 years, respectively.

8.5. FluINsure™ intranasal vaccine (Louis Fries, ID Biomedical Corporation, Baltimore, MD)

FluINsure™ is a trivalent, subunit influenza vaccine for intranasal inoculation that is composed of inactivated, partially-purified influenza antigens in non-covalent complex with the Proteosome™ adjuvant/delivery system. The vaccine incorporates influenza antigens and *Neisseria meningitidis* outer membrane proteins (OMPs; predominantly porin B, otherwise known as class II OMP). The processing of the Proteosome™ proteins leaves <0.1% nucleic acids and <2% *N. meninigitidis* lipopolysaccharides in the final product. The process has been shown adaptable to egg-grown, detergent-split antigens inactivated by either formalin or β-propiolactone, and can also be used with recombinant antigens generated in a baculovirus system. The FluINsure™ vaccine is stable by mouse immunogenicity and HA quantitation by single radial immunodiffusion (SRID) assays for ≥1 year at 2–8 °C, and at least 3–6 months at room temperature. Although initially formulated with thimerosal, a preservative-free, 0.22 μm filterable product packaged in a unit dose device is under development.

In mice, nasally-administered FluINsure™ induces serum IgG and serum HAI antibody responses equal to, or greater than, the same doses of the influenza antigens alone given IM. In addition, intranasal FluINsure™ induces nasal and lung wash virus-specific IgA, whereas minimal IgA is detected following IM vaccine. While intranasal influenza antigens alone may induce some secretory responses, FluINsure™ is 1–2 orders of magnitude superior. In addition, FluINsure™ is associated with IgG1: IgG2a ratios suggestive of a Th1-shifted response similar to virus infection. This correlates with the observation that spleen cells of animals immunized with nasal FluINsure™ produce the same amount of IFN-γ as those of animals immunized with IM vaccine when restimulated with virus in vitro, but much less IL-5 (a Th2 cytokine).

FluINsure™ can protect mice against a lethal respiratory challenge with mouse-adapted homologous virus in a manner indistinguishable from IM vaccine, and also protects ferrets against fever and/or virus shedding following

challenge with recent epidemic strains. Animal toxicity studies have demonstrated no product-related clinical toxicities or no CNS (including olfactory bulb) pathology.

A total of 331 adults have been enrolled in five clinical trials of monovalent prototypes or trivalent FluINsure™ to date (248 active; 83 placebo or control). Monovalent prototype and trivalent data are highly consistent regarding both immunogenicity and safety. There have been no significant safety issues. The only reactogenicity significantly related to the vaccine formulation has been short-lived ($\leq$2 days median), mild nasal congestion and clear, scanty rhinorrhea. Such nasal symptoms have occurred in about 40–50% of vaccinees. No subject receiving active treatment has had fever or hypersensitivity. All FluINsure™ regimens containing at least one dose of $\geq$15 μg of influenza HA have induced a three- to five-fold increase in serum HAI G.M.T. among subjects previously susceptible (HAI < 40) to the virus in question; and 45–65% of such subjects sero-convert by day 28. All regimens tested have induced at least transient rises in nasal antibody levels. Single-dose regimens induce approximately two-fold increases in geometric mean levels, and two-dose regimens induce 2.5–4-fold increases. With the exception of the lowest single-dose regimen, all nasal antibody levels have persisted at elevated geometric mean levels during a 3–4-month follow-up.

Overall, immune responses to influenza A/H1N1 and A/H3N2 antigens have been quite consistent across multiple studies and lots, whereas responses to B antigens (as with many influenza vaccines) are more variable. In trivalent studies performed to date, potentially protective immune responses have been seen in 70–80% for A/H1N1 strains, 70–90% for A/H3N2 strains, and 40–100% for B strains.

In summary, the FluINsure™ proteosome intranasal influenza vaccine appears to be a promising candidate as it is inexpensive and easily adaptable to new viruses. It appears to be safe and immunogenic in animals and humans. Further controlled studies of the protective efficacy of this vaccine are ongoing.

8.6. Virosomes in influenza vaccination (Reinhard Glück, Berna Biotech Ltd., Berne, Switzerland)

One commercial parenterally administered virosomal influenza vaccine, called Inflexal V, was licensed in Switzerland in 1997 and is currently available in 25 countries worldwide. Studies in adults have demonstrated that the Inflexal V is equal to conventional inactivated influenza vaccine (Fluarix) in terms of immunogenicity and appears to be better tolerated. A new virosomal vaccine, adjuvanted with MF59 is currently under development. The adjuvanted vaccine has been tested in HIV+ and HIV− children. Immune responses are more frequent in HIV− children. Among the HIV+ children, sero-protective serum antibody responses were documented in 82, 57, and 61% for A H3, A H1, and B, respectively, while seroconversion was noted in 74, 57, and 52%, respectively.

Finally, a non-virosomal intranasal influenza vaccine that used *Escherichia coli* heat-labile toxin as an adjuvant, previously manufactured by Berna, was removed from the market because of a possible association with Bell's Palsy. An epidemiologic investigation compared the frequency of Bell's palsy in 10,000 commercial IM vaccinated patients and 10,000 Berna IN vaccinated patients. Two of the IM and five of the IN patients had Bell's Palsy. Although the incidence was low, the company decided to remove the product from the market.

8.7. Subunit RSV vaccines (Valerie Sales, Aventis Pasteur, Toronto, Canada)

A RSV-A subunit vaccine that contains the F, G, and M proteins formulated with an alum adjuvant for intramuscular injection has been developed recently. The target population for this vaccine will be the elderly and high-risk adults. Preliminary studies of the immunogenicity and tolerability have been undertaken in 40 healthy adults aged 18–45 years. Of 30 randomized to active vaccine, 24 were evaluable at 14 months, while 9 of 10 who were randomized to an alum-placebo group were evaluable. The most frequent adverse effects from the vaccine included pain and tenderness that tended to resolve on its own within 72 h. The data on the anti-F and anti-G antibody titers are summarized in Table 3. Although titers declined over time, the level at 14 months in vaccinated individuals was consistently above the baseline. This study showed that the vaccine was safe and immunogenic, although annual vaccination would likely be required to maintain protective antibody levels. A follow-up study gave active vaccine to 12 vaccinees and 7 placebo

Table 3
Immunogenicity of an RSV-A subunit vaccine

		Log_2 geometric mean titer			% With four-fold increase in titer	
		Baseline	1 month	14 months	1 month	14 months
Vaccine	NA to RSV-A	11.2	14.3	12.1	83.3	20.8
	NA to RSV-B	10.8	13.6	11.7	76.7	16.7
Placebo	NA to RSV-A	10.9	10.6	10.2	0	0
	NA to RSV-B	9.8	9.8	9.6	0	0

NA: neutralizing antibodies.

Table 4
Immunogenicity of an RSV-A subunit vaccine given 14 months after the first vaccine

		Log_2 geometric mean titer		% With four-fold increase in titer
		Baseline	1 month	1 month
Second dose (active vaccine year 1)	NA to RSV-A	2.0	13.1	25.0
	NA to RSV-B	11.4	12.3	8.3
First dose (placebo year 1)	NA to RSV-A	10.1	13.0	85.7
	NA to RSV-B	9.1	11.8	71.4

NA: neutralizing antibodies.

recipients from the first study at 14 months after their initial vaccination. As shown in Table 4, antibody titers decreased over time. Revaccination was immunogenic suggesting that annual vaccination may allow the maintenance of protective antibodies. Further clinical trials with this vaccine are underway.

8.8. Recombinant hybrid RSV vaccine (Ultan F. Power, Centre d'Immunologie Pierre Fabre, Saint-Julien-en-Genevois, France)

BBG2Na is a recombinant chimeric protein comprised of BB (the albumin binding domain of Streptococcal protein G) and G2Na (residues 130–230 of the RSV-A Long strain G protein) expressed in *E. coli*. In pre-clinical studies, we found that BBG2Na formulated in alum was immunogenic and protective against RSV-A and -B challenges (Power et al., 1997), was immunogenic and protective in murine neonates, even in the presence of high anti-RSV maternal antibody titers (Brandt et al., 1997; Siegrist et al., 1999), did not induce evidence of enhanced pathology (Corvaia et al., 1997; Plotnicky-Gilquin et al., 1999b; Power et al., 2001a), and protected via antibodies and $CD4^+$ T cells (Plotnicky-Gilquin et al., 1999a, 2000; Power et al., 2001b).

A dose-escalating phase I study in 108 healthy young adults, aged 18–45 years old, demonstrated that BBG2Na + alum was well-tolerated and immunogenic, as demonstrated by specific antibody responses following intra-muscular (IM) injection (Power et al., 2001b). Indeed, 63–100% of subjects from each of the three vaccine groups had more than two-fold IgG antibody rises to G2Na. Furthermore, 33–71% of subjects had more than two-fold increases in virus neutralization (VN) titers after 100 and 300 μg doses. Doses of 100 and 300 μg demonstrated similar immunogenicity, while 10 μg induced lower responses. Apart from slight increases in VN titers following second dose, there was no apparent advantage of giving more than 1 dose. The vaccine resulted in no severe adverse events, but 15–22% of patients had mild pain, redness, or edema at the injection site and 2.5% developed a fever following vaccination.

A phase II study in 307 ambulatory elderly adults, aged 60–80 years, was undertaken to determine the dose regimen of BBG2Na to be used for phase III efficacy trials. Patients were randomized, in a blinded fashion, to doses of 50, 100 or 200 μg or placebo administered IM either once or twice at 4-week intervals. The most frequent adverse effects of the vaccine were local reactions that consisted mainly of pain and to a much lesser extent, local redness, induration and edema. Oral temperatures of >37.5 °C were observed in four individuals, while there was a tendency to have more reactions with the highest vaccine dose and with a second injection. One case of "flu-like syndrome" was recorded with a transient rise of hepatic enzymes. Two cases of hypersensitivity type III-like reactions were also recorded. Both were rapidly resolved within a few days with no sequelae. The overall tolerance profile was favorable, and an independent Experts Board recommended that clinical development should be continued, but with increased safety documentation in the phase III efficacy study.

As expected, antibody responses were lower in the elderly than those previously observed in healthy young adults. Nonetheless 50–70 and 35–50% of subjects demonstrated at least two- and four-fold rises in anti-G2Na antibody titers, respectively, at 1 month post-vaccination, depending on the group. Furthermore, >30% of subjects also demonstrated two-fold VN titer increases. There was no dose–response evident between the three doses tested, and no boost was evident following the second dose. During follow-up, GM antibody titers dropped progressively, such that by 12 months most subjects returned to baseline titers.

In conclusion, these data indicate that BBG2Na + alum is immunogenic in the elderly and that a single dose regimen probably suffices. However, safety monitoring would be an essential component for the phase III efficacy studies.

8.9. Current status of RSV live attenuated vaccine (Peter Wright, Vanderbilt University Medical Center, Nashville, TN)

RSV affects 75% of children during their first year of life and is responsible for over 100,000 hospitalizations and 500 deaths per year in children. The additional costs attributable to RSV infection has been calculated to be over US$ 300 million per year. Although 53% of children have no risk factors, many children that have infections severe enough to require hospitalization were born premature, had congenital heart disease, or bronchopulmonary dysplasia. RSV causes illness from December to March and accounts for 4% of URIs among community-dwelling children and 17% of LRIs. To help combat this disease, a cold-passage,

live-attenuated vaccine was created using reverse genetics. The resultant 248/404/ΔSH temperature-sensitive, attenuated virus has been used to vaccinate unscreened adults, RSV seropositive children 15–59 months old, RSV seronegative children 6–24 months, and 1–2-month-old infants. After a 10^5 initial dose, only 1/15 adults and 1/10 seropositive children had recoverable virus. Of those with recoverable virus, the titer was quite low (1.6 $\log_{10}$). Seronegative children shed virus in nasal secretions with a peak viral titer of 2–4 $\log_{10}$; most of the seronegative children ceased shedding within 12 days.

An alternative vaccine, the 248/404/1030/ΔSH virus was also tested and found to have lower rate of infection and titer of shedding. Additional testing with these attenuated strains showed that increasing the initial vaccine inoculum titer does not substantially increase viral shedding or immunogenicity. Finally, studies in children less than 3 months old showed a comparable level of viral shedding in the presence or absence of maternal antibodies. The young infants had minimal antibody responses, although there were detectable serum levels of IgG and IgA to the G protein. Mucosal antibodies were produced but at far lower levels than are seen in children older than 3 months of age. A second dose of vaccine, the response to which can be used as a surrogate for reinfection, resulted in rare shedding of the virus and no evidence of enhanced RSV illness once infected. These findings suggest that the vaccine may be protective without detectable increases in antibody level. Finally, a NS-2 protein deleted 248/404 virus, a change that may make the virus more susceptible to interferon, has been found to have markedly reduced viral shedding, particularly in seronegative children, when compared to the other attenuated vaccine strains. Although the study of these live-attenuated vaccines against RSV have given limited information about efficacy, they have given significant data to support continued develop of these vaccines.

8.10. Parainfluenza virus type 3 vaccines (Ruth Karron, Johns Hopkins University, Baltimore, MD)

Parainfluenza virus type 3 is a frequent cause of infection in young children. One study found that 100% of children 0–12 years of age had experienced PIV3 infections and that ∼60% of children age 13–24 months and ∼35% of children age 25–36 months were later reinfected with the same strain, suggesting incomplete protection following primary infection (Glezen et al., 1984). Two different PIV3 vaccines are currently under development with the goal of preventing PIV-associated lower respiratory tract illness in those at risk: a live-attenuated bovine vaccine, which is being developed by MedImmune and NIH, and a cp45-based vaccine that is being developed cooperatively by Wyeth and NIH.

Although bovine PIV3 was well tolerated when used to vaccinate infants and children, only 70% of vaccinated children developed a four-fold rise in antibody titer, far less than would be seen with natural HPIV3 infection. The reciprocal mean pre- and post-vaccine HAI titers to HPIV3 test antigen were 2 and 21, respectively, in BPIV3 vaccinees and 4 and 104, respectively, in hPIV3 vaccinees. To determine which components of the BPIV3 were responsible for attention, reverse genetics were used to create an array of hPIV-3 variants with single bovine gene replacements. All of the recombinant strains resulted in improved antibody response and afforded protection against hPIV3 relative to BPIV3 in monkeys. Two chimeric viruses, rHPIV3-N_B and rBPIV3-F_H-HN_H are felt to be the strongest candidates and plans are being made to test these viruses in human. Additional potential uses of these recombinant PIV-3 viruses are as vectors to express PIV-1 and PIV-2 HN and F proteins, RSV F and G proteins, measles H protein, or surface glycoproteins of other respiratory viruses (Bailly et al., 2000; Durbin et al., 2000; Schmidt et al., 2000, 2002; Skiadopoulos et al., 2001; Tao et al., 2000).

Another attenuated virus that is being developed for PIV3 vaccines is the cp45 HPIV3 virus grown in Vero cells. Serial passage at low temperatures in AGMK has resulted in a virus that is cold-adapted, temperature-sensitive, and attenuated in human and non-human primates. As a result, the virus does not grow well at temperatures below 37 °C, a characteristic that theoretically limits its replication to the upper airway. In phase 1 studies in adults and seropositive children 1–59 months, intranasal inoculation of 6 $\log_{10}$ pfu resulted in infection in only 10% of adults and 12% of the seropositive children. This suggests that prior natural HPIV3 infection limits the infectiousness of the vaccine virus. In a phase 2 study of seronegative 6–24-month children given either 4 or 5 $\log_{10}$ pfu, 82–100% were successfully infected. Of those infected, the mean peak titer of shed virus was 2.2–3.3 $\log_{10}$ pfu/ml. In these studies, the vaccine was well tolerated without significant adverse effects, although a small number of URIs and an increased number of otitis media episodes were observed in the phase I but not phase II studies.

Next, a two-dose study in 1–2-month-old infants was conducted, with the second dose being used as a surrogate challenge infection. The first dose of vaccine was well tolerated; similar frequencies of fever, congestion, LRI, and OM occurred in vaccinated and unvaccinated children. When children were revaccinated a month after their first exposure, there was much less shedding of vaccine virus with the second dose. In patients who received their second dose 3 months after the first, virus shedding was detected with greater frequency than after the 1-month challenge. Vaccination resulted in a significant rise in serum IgA anti-HN in 84% of children but did not result in significant HAI titer changes. Five children had temperature-sensitive intermediate viruses recovered during follow-up. All five formed plaques at 39 °C, represented a small fraction (1%) of the total viral population, presented at the peak of viral shedding and were cleared at the same rate as fully temperature-sensitive viruses, and were not associated with changes in clinical status. From these studies, it is clear

that the cp45 HPIV3 vaccine is well-tolerated and immunogenic in infants and children but uncommonly may partially revert. Multiple doses will be needed in infancy and the interval between doses should not be more than 2 months.

As a result of these positive findings, an RSV-A cpts 248/404 vaccine was tested alone and in combination with the HPIV-3 cp45 vaccine in a randomized, double-blind study in 6–18-month-old healthy children dually seronegative to RSV (neutralizing antibody $<$ 1:40) and PIV3 (HAI $<$ 1:8). Children were given either RSV, HPIV3, both, or placebo vaccines containing 1×10^5 pfu of each vaccine virus in 0.5 ml. Fever occurred in 17, 8, 33, and 44% and otitis media in 33, 8, 33, and 11% of the RSV, HPIV3, both, and placebo groups, respectively. All four children with a fever above 38 °C were shedding both RSV and PIV3. The percent infected was 92% for both RSV and PIV3 when used as the sole vaccine, but in those given the combined vaccine, 90% were infected with RSV while only 76% were infected with PIV3. The combined vaccine induced lower titers of PIV3 antibody than cp45 virus given alone. The antibody responses to the vaccines are listed in Table 5. In conclusion, the majority of children responded to both RSV and PIV3 in the combination vaccine, but interference between the two virus vaccines was evident in some children. Further studies are planned to investigate the incidence of OM and the utility of two doses of both PIV3 and combination vaccines.

8.11. Local immune response and efficacy after vaccination of children 3–6 years old with Russian live influenza vaccine (LIV) (Julia Desheva, Institute for Experimental Medicine, St. Petersburg, Russia)

A live-attenuated vaccine (LIV) that was created using genetic reassortment of epidemic strains into three attenuated master strains (A/Leningrad/134/17/57 (H2N2), A/Leningrad/134/47/57 (H2N1), and B/USSR/60/69). The current trivalent vaccine contains A/H1N1, A/H3N2, and B influenza strains. The intranasally administered vaccine has been shown previously to be safe and well tolerated in adults and children. Children typically receive two doses of the vaccine, 2 weeks apart, while adults get a single vaccine. To investigate the response to this vaccine in children age 3–6 years old, an open-label study enrolled 369 children over two influenza seasons to receive the intranasal trivalent LIV. Patients were interviewed after 6 months to assess for acute respiratory infections. The vaccine was well tolerated with children uncommonly developing fever (0.9%), headache (0.3%), and sore throat (0.9%). There was a four-fold rise in serum influenza-specific antibodies in 58–59, 58–61, and 30–39% of children to H1N1, H3N2, and B antigens, respectively and four-fold rise in IgA titers in 44–60, 42–53, and 22–44% of children, respectively. There was a 8.8-fold reduction in influenza infections and a 2.2-fold reduction in all acute respiratory infections among vaccinated children compared to historical controls. Likewise, the vaccine was 60% effective in preventing otitis media and 100% effective in preventing bronchitis. This LIV that is currently used in Russian appears to be safe, well tolerated, and effective in children 3–6 years old.

Table 5
RSV/PIV3 combination study antibody response to vaccine

Vaccine group	Number with rise in antibody level		
	RSV	PIV	Both RSV and PIV
RSV	9/10	2/11	1/10
PIV3	2/12	9/11	2/11
RSV and PIV	18/19	12/20	11/18
Placebo	0/5	3/9	0/5

8.12. Reactogenicity and antigenicity of influenza A/Hong Kong/1073/99 H9N2 subunit and whole virus vaccine in healthy adults (Maria Zambon, Public Health Laboratory, London, UK)

Following a highly pathogenic avian influenza A H5N1 outbreak in Hong Kong in 1997, enhanced surveillance identified widespread prevalence of H9N2 virus in Asian swine herds and live-bird markets. In 1999, two children developed influenza caused by a H9N2 strain in Hong Kong. The H9N2 virus exhibits receptor-binding properties consistent with other human influenza viruses suggesting that this virus could potentially cause a pandemic. An inactivated subunit H9N2 vaccine was protective in mice. Since whole virus vaccines may be more immunogenic in naïve patients, whole virion (WV) and subunit (SV) vaccines were prepared using influenza A/Hong Kong/0173/99 (H9N2). This vaccine was used in an observer-blind, dose-ranging study in 60 healthy adults aged 18–60 years old. Volunteers received either vaccine containing 7.5, 15, or 30 μg H9 HA at the time of enrollment and 21 days later. Antibody response at days 0, 14, 21, and 42 was detected by hemagglutination inhibition (HI), microneutralization (MN), and single radial hemolysis (SRH). Both vaccines were well tolerated, although the WV was associated with more frequent pain (42% versus 10%), nausea (15% versus 0%), and arthalgias (15% versus 3%) than the SV. There were no differences in seroconversion between WV and SV at day 42, although seroconversion was more likely in patients who had received 30 μg, particularly with the WV. Patients younger than 32 years were less likely to convert after the first dose than following the second dose. This suggests that a booster dose will be required for all naïve patients younger than 32 years.

9. Antivirals and treatment

9.1. Treating common colds: Echinacea and beyond (Ronald Turner, University of Virginia, Charlottesville, VA)

Echinacea is one of the most widely used herbal remedies for the common cold. Despite its frequent use and the

Table 6
Biologically active constituents of the three species

Species	Cafeic acid derivatives	Polysaccharides	Alkamides
E. angustifolia	Echinacoside	Present	Monoenes
E. pallida	Echinacoside	Trace	Ketoalkenyns
E. pupurea	Cichoric acid	Present	Dienes

myriad of preparations, the herb is regulated in the United States as a dietary supplement and is therefore not held to the same standards as pharmaceuticals. *Echinacea* is derived from three different medicinal herb species: *Echinacea angustifolia*, *E. pallida*, and *E. purpurea*. Antiviral effects are attributable to the caffeic acid derivatives cichoric acid and echinacoside, in addition to polysaccharides and alkamides present in the plant. Immunostimulatory activity has been ascribed to the polysaccharides, while anti-inflammatory activity has been ascribed to the caffeic acid derivatives and the alkamides. The biologically active constituents differ among the three species (Table 6). Unfortunately, the content of each biologically active component in the plant is dependent on the harvest time and part of the plant used. Available preparations use different species, different parts of the plant, and plants from different regions and harvest times. As a result, there is significant variability in the content of cichoric acid and alkamides in different brands and different lots of the same brand. Studies of these agents are difficult because it is unclear what are the optimal dose and formulation of *Echinacea* to use and what are the relative absorption and distribution of each preparation.

To prospectively study *Echinacea* for the common cold, a standardized *Echinacea* extract, containing 0.16% cichoric acid and no echinacosides or alkamides was prepared. Sixty-three patients ingested the active agent and 54 placebo for 2 weeks prior to challenge with rhinovirus type 23. Rhinovirus infection occurred in 44% of *Echinacea*-treated and 57% of placebo-treated volunteers, an insignificant difference. Likewise, there was no difference in symptoms between the two groups. This preparation of *Echinacea* did not prevent or lessen the severity of rhinovirus colds. In summary, activities of *Echinacea* extracts are unclear, the products are not standardized, and the currently available studies provide limited data that can be generalized to different products.

Components of the pathogenesis of rhinoviral colds need to be considered as targets for therapeutic intervention. Virus infection of the nasal epithelium results in cellular oxidative stress and in a non-specific host inflammatory response that results in sore throat and vasodilation and serum transudation. These events in turn result in nasal obstruction and rhinorrhea. Progression of the virus to the lower respiratory tract may also occur. One step amenable to modification may be the oxidative stress induced by the virus. In A549 cell lines exposed to RSV, exposure to the antioxidant DMSO resulted in reduced IL-8 production in a concentration-dependent manner. To investigate the role of oxidative stress in rhinovirus infections, carbonyl and DCFDA stains were used to document oxidant stress in cell lines exposed to rhinovirus. Next, *N*-acetylcysteine (NAC), another potent anti-oxidant, was used in BEAS-2B cell lines exposed to hRV. NAC exposure was associated with significant decreases in NFκB and IL-8 in a concentration-dependent manner.

To further elucidate the role of various agents in the management of rhinoviral colds, anti-ICAM antibodies were studied to determine their impact on cytokine production. Anti-ICAM exposure of cell culture was associated with a significant decrease in viral titer, but there was no difference in IL-8 production in wells exposed and unexposed to anti-ICAM, suggesting that elaboration of IL-8 is independent of viral binding to ICAM.

Several oxidase inhibitors, including diphenylene iodonium (DPI), ibuprofen, allopurinol, and rotenone were tested in a similar cell culture system to determine their impact on rhinoviral replication and IL-8 production. Only DPI appeared to reduce viral titers and IL-8. Additional testing found that DPI was successful in reducing IL-8 production in response to RSV and coronavirus infection as well. Since DPI is a specific inhibitor of NADPH-oxidase, additional studies were conducted to determine the components of the oxidase most responsible for the reduction of titers. First, CGD skin fibroblasts, which are deficient in the gp91-*phox* component of the NAPDH-oxidase, were infected with hRV. There was no difference in viral titer or IL-8 production as compared to normal skin fibroblasts, while fibroblasts that were p47-*phox* deficient had reduced IL-8 production. Lastly, p47-*phox* antisense molecules were used to assess the role of p47-*phox* in IL-8 production. In the presences of antisense constructs, IL-8 levels were markedly reduced. Amplification of MRC-5 and BEAS-2b cell lines to look for specific NADPH-like oxidases found NOX-4 to be present in all tested cell lines. Future studies will be directed at specific inhibitors of NOX-4 to assess their ability to limit the viral titer and symptoms as the result of these infections.

9.2. Inhibition of human rhinovirus infection by a tetravalent anti-ICAM 1 Fab fusion protein, CFY196 (Fang Fang, Perlan Therapeutics, San Diego, CA)

Over 90% of human rhinovirus (HRV) use ICAM-1 as the receptor to attach to respiratory epithelium (Greve et al., 1989; Tomassini et al., 1989). In the past, two different compounds have been developed to bind to either ICAM-1 or to block ICAM-1 binding. Soluble ICAM-1 was found to prevent and treat experimental HRV infections in adults. Unfortunately, it had to be given in high doses six times a day to be effective, and has not been pursued for clinical development (Huguenel et al., 1997; Turner et al., 1999). A murine monoclonal antibody, termed RRMA, which blocked ICAM-1, was found to be safe in humans and partially effective in preventing experimental hRV infection in one trial (Hayden et al., 1988). Our in vitro investigation of RRMA

showed that its kinetics with respect to receptor interactions were not favorable. In particular, the k_{on} was 2.4×10^{-3} and $(17–47) \times 10^{-3}\,s^{-1}$ for HRV and RRMA, respectively, whereas the k_{off} was 1.67×10^{-3} and $1.80 \times 10^{-3}\,s^{-1}$, respectively. These kinetics showed that RRMA avidity was insufficient to compete effectively against HRV.

Since multivalency of antibodies increased binding affinity by decreasing k_{off}, we constructed a tetrameric recombinant RRMA antibody. Each of the four arms of the molecule, called CFY196 (ColdSol^{TM}), has three domains: a humanized RRMA Fab, a flexible linker, and a tetramerization domain. The gene for this antibody construct was used to transform *E. coli* to produce a homogenous tetramer after ultracentrifugation. Binding kinetic studies were done on cells with CFY196 and the k_{off} was $1.53 \times 10^{-6}\,s^{-1}$, and the binding half-life was greater than 80 h. In vitro experiments using HeLa and primary human lung cells has demonstrated that nanomolar concentrations of the drug are effective at preventing HRV replication and cytopathic effect. The molecule is stable for over 3 months at 37 °C and for over a week in nasal mucus at 37 °C. As a result of these promising studies, the compound is being developed as an intranasal anti-rhinovirus agent. Additional preclinical studies are ongoing and plans for clinical trials are being developed.

9.3. Cyanovirin-N exhibits anti-influenza activity through binding to viral hemagglutinin and this ability is greatly reduced in mouse-adapted viral strains (Donald Smee, Utah State University, Logan, UT)

Cyanovirin-N (CV-N) is a 101 amino acid protein isolated from the cyanobacterium *Nostoc ellipsosporum* with antiviral activity against the human immunodeficiency virus (Chang and Bewley, 2002). CV-N is active in vitro against influenza but has little or no activity against adenovirus, parainfluenza virus, respiratory syncytial virus, enterovirus, rhinovirus, or coxsackie virus.

Inhibition is strain-dependent and several strains of influenza A (H1N1) and influenza B were highly resistant to CV-N. Much less CV-N binds to resistant A/PR/8 than sensitive A/Sydney virus, consistent with the antiviral activity observed in cell culture. These studies led to binding experiments using purified HA from an A/Beijing (H1N1) virus. Europium-labeled CV-N bound to viral HA that contained high mannose residues, and exogenous oligomannose-8 could inhibit the binding of CV-N to the HA.

In virucidal assays, in which pools of stock virus were incubated with CV-N for 30 min prior to virus titration on cells, found that A/NWS and A/PR/8 viruses were resistant to virucidal effects. Mouse-adapted viruses (A/Shangdong, A/Duck, A/Gull, and B/Hong Kong) were much more resistant to the compound than cell-culture passaged viruses. The A276T change found in mouse-adapted A/Shangdong resulted in a loss of the high mannose glycosylation site at that position on the viral hemagglutinin, thus is responsible for viral resistance to CV-N.

In conclusion, interaction between CV-N and the influenza hemagglutinin is carbohydrate-mediated, but CV-N discriminately binds only the glycoproteins that express high-mannose (Man-8 and Man-9) oligosaccharides. Virus resistance to CV-N correlated with loss of glycosylation sites in the viral hemagglutinin.

9.4. Ruprintrivir (AG7088) (Amy Patick, Pfizer Global Research and Development, La Jolla, CA)

In the search for drugs active against picornaviruses, the 3C protease was identified as a important target because it lacks similarity to human proteases, is essential for replication, and has a highly conserved active site (Matthews et al., 1994). 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; Patick et al., 1999). Phase I trials demonstrated that ruprintrivir had minimal treatment-related adverse nasal effects and that there were high nasal mucus concentrations of ruprintrivir 12 h after intranasal inoculation. In phase II studies, in which volunteers were inoculated with either HRV39 or Hanks, subjects were randomized to receive intranasal ruprintrivir (8 mg) or placebo sprays as prophylaxis (2 or 5 times daily (2 times per day or 5 times per day) for 5 days) starting 6 h before infection or as treatment (5 times per day for 4 days) starting 24 h after infection. Patients who received active drug as treatment had lower symptom scores beginning 1 day after infection. The treated patients also had lower viral titers by culture and RT–PCR at days 2 and 3. Prophylaxis also reduced viral shedding and mean AUC of virus titer but did not significantly reduce the frequency of colds. These studies established that intranasal ruprintrivir suspension has significant antiviral activity in experimentally induced rhinovirus infection. A phase II treatment trial of patients with natural colds with symptoms of less than 36 h was conducted in which patients received ruprintrivir BID or TID or placebo for 10 days. Unfortunately, only 29% of volunteers had a picornavirus documented by a first generation RT–PCR test and the study found no difference in outcomes between treated and untreated patients, except for those picornavirus-infected patients who entered the study within the first 24 h of symptomology. As a result of these results the drug was reformulated to its current 2% nasal solution. Phase I trials showed the drug to be safe and well tolerated; phase II studies are currently underway.

9.5. Pleconaril (Mark McKinlay, ViroPharma, Exton, PA)

Pleconaril is an orally bioavailable capsid-inhibitor that blocks uncoating and attachment of picornaviruses by binding into a hydrophobic pocket within the capsid. It has broad activity against most picornaviruses. Two pivotal phase three clinical trials were conducted from August to November 2000 at 197 centers in the United States and

Canada. Patients that had an afebrile cold with symptoms of less than 24 h with moderate to severe rhinorrhea were enrolled. Patients received pleconaril 400 mg BID for 5 days after enrollment. A total of 2096 patients were enrolled in both trials with 65% having a rhinovirus cold by RT–PCR. Pleconaril treatment was associated with a reduction in illness duration of 0.6 and 1.5 days in each study, respectively (Hayden et al., 2003). Illness severity was reduced by 19% and differences began on day 2. Nights of sleep disturbance was reduced by 33%, cold medications were reduced by 19%, and viral shedding was reduced by 2 days. Resistant virus was isolated post-treatment in some subjects; 2.7% showed no susceptibility to pleconaril and 10.7% had a 10-fold reduction in susceptibility. Amino acid changes from resistant variants mapped to the drug-binding pocket. There was no difference in illness course in patients shedding virus with reduced susceptibility to pleconaril. Mild to moderate nausea was more common among patients who received pleconaril (6% versus 4%). There were no clinically significant effects on laboratory safety parameters. Three-and-a-half percent of women on oral contraceptives developed menstrual disorders after using pleconaril, although there was no increased incidence of pregnancy in these women. In conclusion, pleconaril was found to be the first antiviral drug effective in reducing the duration and severity of rhinoviral colds and was well tolerated.

A 6-week prophylaxis study was then launched which enrolled 1069 adults who were treated with pleconaril 400 mg BID, 400 mg QD, or placebo for 6 weeks. The incidence of picornavirus-confirmed colds was 8, 9, and 16% for pleconaril BID, QD, and placebo, respectively. Treated patients also had a corresponding approximately 50% less functional impairment, sleep disturbance, and missed days from school or work when they were receiving pleconaril versus placebo. The safety profile of pleconaril in this study was similar to the treatment study, although there was a two- to three-fold increased rate of menstrual disorders in pleconaril recipients compared to placebo. The increase in events began during the second week of prophylaxis. In response to these findings, additional testing was done in healthy volunteers that documented increases in CYP3A activity after 5 days of oral pleconaril. This increased CYP3A activity was associated with a 35% decrease in the AUC of ethinyl estradiol and a 28% decrease in the AUC of midazolam. The CYP3A activity returned to normal levels 7–14 days after cessation of pleconaril administration. It is interesting to note that there was no evidence that pleconaril had an impact on the cytochrome P450 system in preliminary studies in rat, dog, or human hepatocyte assays.

Although the FDA Advisory Panel that reviewed the application for pleconaril agreed that it was efficacious, there were concerns about drug interactions, development of antiviral resistance, its probable over-the-counter-like use if approved, and the generalizability of the available efficacy data to higher risk persons. As a result of these concerns, the FDA deemed pleconaril not approvable in June, 2002. Currently, pleconaril is still available as part of a compassionate use program, and ViroPharma is pursuing approval of the oral dosage form for life threatening enteroviral infections, including chronic meningoencephalitis, myocaridits and neonatal sepsis. In addition, there is hope to reformulate pleconaril for intranasal delivery which will hopefully allow delivery the drug to the site of infection without allowing systemic absorption with its resultant risk of inducing the CYP3A system.

Currently, pharmaceutical interest in compounds with activity against respiratory viruses is limited because of a focus on chronic disease, unattractive commercial opportunities, and the high standard required to obtain FDA approval for non-life-threatening conditions. Despite these limitations, colds are the most common reason for seeing a physician and result in nearly 75% of patients taking over-the-counter medications or antibiotics. Antiviral agents with activity against rhinoviruses are needed and may have additional benefits such as reducing the indiscriminant use of antibiotics with its resultant induction of antimicrobial resistance.

9.6. Update on RSV fusion inhibitors (Dan Pevear, ViroPharma, Incorporated, Exton, PA)

As has been described earlier, RSV is an important pathogen in infants, the elderly, and the immunocompromised and is responsible for over 100,000 excess hospitalizations and US$ 2 billion in direct medical costs annually. Several compounds are under development that may be effective in treating RSV infections. One such class of compounds inhibits RSV replication by affecting functions associated with the viral fusion (F) glycoprotein: Janssen's R170591, a topically applied compound with a reported EC_{50} against RSV of <1 nM, is in preclinical study; Bristol-Myers Squibb's BMS-433771, an orally bioavailable compound with a reported EC_{50} against RSV of 10 nM, is in preclinical study; and ViroPharma's VP14637, a compound delivered by small particle aerosol (SPA) inhalation with an EC_{50} against RSV of <1 nM, is completing phase 1 studies.

VP14637 is broadly active in cell culture against RSV-A and -B viruses. In vitro time of drug addition studies suggest that the drug has to be present early after infection to affect a single round of virus replication. In cotton rats, prophylactic BID dosing of VP14637 by SPA for 3 days resulted in significant reduction in lung virus titer. Forty-six independent escape variants were selected to VP14637 in vitro. All 46 variants had amino acid changes that mapped to the F glycoprotein. Modeling of these changes on the crystal structure of the Newcastle Disease Virus metastable form of the F glycoprotein indicated that the amino acid changes mapped to the head domain of the protein (Chen et al., 2001). Preclinical toxicology studies in adult rats, adult dogs, and neonatal dogs in which the animals were given VP14637 for 2 weeks by SPA inhalation resulted in no histological changes. Genotoxicity studies have been negative to date. An IND for the new drug was filed and approved during the third quarter

of 2001. A phase 1 study has recently been completed, although data analysis is ongoing, and other studies are in the planning stages currently.

9.7. Development of a novel cell line with enhanced ability to detect neuraminidase inhibitor resistant variants (Mikhail Matrosovich, Philipps University, Marburg, Germany)

Influenza virus can develop resistance to neuraminidase inhibitors via changes in the neuraminidase (NA), which results in decreased drug binding, or in the hemagglutinin (HA), which results in lowered HA affinity for its cellular receptor. To detect both types of resistance, and perhaps novel ones, a virus inhibition assay in cell culture is required. Unfortunately, current cell culture assays are not predictive of influenza virus sensitivity to neuraminidase inhibitors (NAI) in enzyme inhibition assays or in vivo (McKimm-Breschkin, 2000; Tisdale, 2000) NAI sensitive strains may appear to be resistant, while NAI resistant mutants may show susceptibility with current methods. One reason for this limitation is that laboratory cell lines do not model influenza virus receptors in the human airway epithelium. Human airway epithelium has mostly two to six linked sialic acids with high receptor density, and influenza virus infection is highly NA dependent. Laboratory cells including commonly used Madin Darby canine kidney (MDCK) cells, on the other hand, have mostly two to three linked sialic acids with low receptor density, so that influenza infection is less NA dependent.

To create a cell line that better reflects normal human airway epithelium, MDCK cells were transfected with β-galactoside a2,6-sialyltransferase (SIAT1). SIAT1 attaches sialic acid at the 6-position of Gal1-4GlcNac. The transfection was achieved using a pIREsneo vector with the SIAT1 gene that resulted in overexpression of SIAT1 and neomycin resistance. Preliminary studies demonstrated that the transfection was stable and resulted in substantially increased two to six linked sialic acid on the cell surface. The cells also had increased binding of human influenza virus compared to standard MDCK cells. In cell culture studies of MDCK and SIAT1-transfected MDCK cells, A/Sydney/5/97 (H3N2), A/Memphis/14/96 (H1N1), and B/Memphis/25/99 were more sensitive to the NAI in the SIAT-transfected cells than the MDCK cells. When wild type A/Wuhan/359/95-like (H3N2), that was isolated before initiation of treatment with a neuraminidase inhibitor, was tested in parallel with a post-treatment E119V NA mutant, the SIAT cells were able to differentiate susceptible from resistant virus, while the MDCK cells were not. Similar results were noted with R292K, N129K, E156K, and Q226R mutants. The drug susceptibility patterns correlated with the affinity for 6-linked receptors of the viruses.

In summary, the SIAT-transfected MDCK cells have acquired genes that are stably expressed and result in increased expression of 6-linked sialic acids and reduced expression of 3-linked sialic acids. Human influenza viruses have improved affinity for these cells and greater sensitivity to NAIs in cell cultures using these transformed cells. The sensitivity of viruses to oseltamivir carboxylate in MDCK-SIAT1 cells was consistent with their sensitivity in NA enzyme assays and with HA receptor-binding phenotype. The SIAT1 transfected MDCK cells appear to be a promising system for testing influenza virus sensitivity to NAIs.

9.8. Sequence variation and susceptibility of influenza virus clinical isolates to zanamivir and oseltamivir prior to their introduction into clinical practice (Jenny McKimm-Breschkin, CSIRO Health Sciences and Nutrition, Parkville, Australia)

Zanamivir and oseltamivir, which specifically inhibit the neuraminidases (NA) of influenza A and B viruses, were introduced into clinical practice in 1999–2000. NA mutations detected in clinical isolates, which confer resistance to the available neuraminidase inhibitors (NAI), include E119Vand H274Y, which confer resistance to oseltamivir only, and R292K and R152K, which confer resistance to both oseltamivir and zanamivir (Wetherall et al., 2003). The Neuraminidase Inhibitor Susceptibility Network (NISN) was established to monitor the potential development of resistance in clinical isolates with the widespread introduction of NA inhibitors into clinical practice (Zambon et al., 2001).

To determine baseline sensitivities of isolates prior to the introduction of drugs, 1054 isolates were collected through the WHO Influenza Surveillance Network from different regions of the world from 1996 to 1999. Sensitivities of the NAs were determined using enzyme inhibition assays. Preliminary testing showed that the MUNANA fluorescent assay (Potier et al., 1979) and NA-STAR chemiluminescent assay (Buxton et al., 2000) gave similar results, although the NA-STAR method was preferred when NA activity was low. There were very few discordant pairs using these two assays. Using these two systems, influenza A/N1 and influenza B viruses tended to be slightly more sensitive to zanamivir, while influenza A/N2 subtypes tended to be slightly more sensitive to oseltamivir (McKimm-Breschkin et al., 2003). Drug susceptibilities of known zanamivir and oseltamivir-resistant mutants fell outside the 95% confidence limits of all natural isolates and were easily distinguishable.

The NAs of approximately 100 isolates, both control viruses and clinical isolates including those lying above the 95% confidence limits, were sequenced. The sequences revealed variation in previously conserved residues. One of 10 N1, 7 of 38 N2, and 1 of 42 influenza B isolates had D151 to N, G, E, or V. Originally the D151 was thought to be the proton donor for the catalytic reaction mediated by the NA. Since none of these isolates showed decreased enzyme activity, the D151 likely does not provide this role. In addition to changes in non-conserved residues associated with natural antigen drift, there was also a change in the conserved residue T225I in 1 B virus, and co-evolution of I203L and E375G in 5/42 B isolates. Molecular modeling

shows these latter two residues lie on opposite sides of the NA head and hence are more likely to be random rather than compensatory mutations. Importantly, despite sequence variations, there was no naturally occurring resistance to either zanamivir or oseltamivir.

9.9. Effect of mutations in the hemagglutinin gene of influenza A viruses on the resistance phenotype of neuraminidase inhibitors (Guy Boivin, Laval University, Québec, Canada)

Previous studies have shown that resistance to neuraminidase inhibitors (NAI) generated after in vitro passages may result in mutations in the neuraminidase (NA) and/or hemagglutinin (HA) genes. However, the effect of HA mutations alone on the resistance phenotype remains to be confirmed in clinical isolates. In influenza A/H1N9 the HA mutation S186F results in 10-fold reduced susceptibility to zanamivir, while S186F + K222T results in 1000-fold resistance to all known NAIs (Blick et al., 1998). The S186F, K222T, and both mutations were incorporated in the HA transcription plasmid using the Quick change site-directed mutagenesis kit (Stratagene). Recombinant influenza A/WSN33 (H1N1) viruses were then generated by reverse genetics (Fodor et al., 1999). In the NA inhibition assay, the wild type and HA mutants had similar low IC_{50} values for zanamivir (0.67–1.06 nM) and peramivir (0.18–0.26 nM). The K222T virus had a three- to four-fold reduction in susceptibility by plaque reduction assay, while the other two mutants were no different than the wild type virus. This data suggests that in vitro generated resistance to NAIs may not be solely attributable to HA mutations as previously reported. In addition, the combined effects of HA and NA mutations needs to be investigated as potential mechanisms for NAI resistance (Nedyalkova et al., 2002). This study also documents the utility of reverse genetics to study various HA substitutions in the same genetic background and the impact of these variant HAs on antiviral susceptibility.

9.10. Recovery of drug-resistant influenza A and B viruses from immunocompromised patients (Larisa Gubareva, University of Virginia, Charlottesville, VA)

Two specific inhibitors of influenza A and B neuraminidase are currently available for clinical use: inhaled zanamivir and oral oseltamivir. Two additional orally active neuraminidase inhibitors (NAI), peramivir and A-315675, are investigational, although there are no current plans to move these agents forward for clinical use. In vitro resistance can be mediated by changes in the hemagglutinin (HA), which is strain-specific, change in neuraminidase (NA), which is both subtype- and drug-specific, both, or by creation of a defective NA. No reliable cell culture-based assays are currently widely available for influenza virus susceptibility testing against NAIs. Drug-selected changes in the NA are detectable by enzyme inhibition assays. These results of these assays are affected if the reaction conditions are varied, even slightly, if there are mixtures of variants present, or if the NA has low activity. Resistance may be defined as a 5–10-fold or greater increase in the IC_{50} values compared to the virus recovered prior to drug exposure. Sequencing of the NA or HA genes can be done to document genetic changes that may be associated with the phenotypic resistance.

Resistant variants have also been recovered in vivo from both immunocompetent and immunocompromised patients. No zanamivir-selected substitutions in either the HA or NA have been recognized in immunocompetent persons. No oseltamivir-selected substitutions in the HA have been recognized. However, several oseltamivir-selected changes in the NA of influenza A viruses have been detected: N1 H274T and N2 G119V and R292K have been found. About 1–2% of treated adults and 6% of children develop these variants during therapy. The clinical course of patients with detectable resistant variants is not different from those with susceptible viruses (Carr et al., 2002; Covington et al., 1999; Gubareva et al., 2001; Herlocher et al., 2002; Whitley et al., 2001).

Immunocompromised patients have prolonged virus shedding which promotes emergence of drug resistance. We have studied four clinical cases of immunocompromised patients who developed NAI resistant mutants have been recognized. The first patient, which has been described elsewhere (Gubareva et al., 1998, 2002) was a 1.5-year-old girl with chronic myelocytic leukemia who underwent bone marrow transplantation. She developed an influenza B virus infection that persisted until her death despite inhaled zanamivir. During zanamivir treatment, a R292K NA mutation which conferred cross-resistance to all known NAIs (IC_{50}: 100 nM zanamivir, 1000 nM for oseltamivir, peramivir, and A-675) developed; in addition T981I, N208D, Q226E, and K325T HA mutations were noted as well.

The second patient was a 2-year-old girl with chronic myelocytic leukemia who underwent bone marrow transplantation. She also developed an influenza B infection that continued until her death. The patient was treated with oseltamivir and briefly with ribavirin. Her initial isolate on therapy had E150G and E273K NA mutations. Over time, additional D198N and D198N NA mutations developed which induced decreasing susceptibility to oseltamivir, zanamivir, and peramivir, but not A-675 (IC_{50}: 304, 15, 5.9, and 1.7 nM, respectively).

The third patient was a 23-year-old male who underwent bone marrow transplantation for acute lymphocytic leukemia. He developed an influenza A/H1N1 infection and received intermittent therapy with amantadine, rimantadine, oseltamivir, and zanamivir and died 18 months later with detectable virus. The initial virus available for resistance assessment, collected after several months of therapy, was resistant to the M2 inhibitors and had V27A, V28D, A30V, L36V, L21I mutations in M2. A H274Y mutation in NA that conferred decreased susceptibility to oseltamivir (IC_{50}: 100 nM) and peramivir (IC_{50}: 10 nM) but no change in

zanamivir or A-675 susceptibility (IC_{50}: 1.1 and 1.2 nM, respectively) was transiently detected.

The fourth patient was a 63-year-old female with chronic lymphocytic leukemia who developed an influenza A/H3N2 infection. Over time, a G119V NA mutation occurred which conferred resistance to oseltamivir only and an S31N M2 mutation conferred M2 inhibitor resistance, so that a virus resistant to both M2 inhibitors and oseltamivir emerged.

From these patients, it is clear that antiviral therapy failed to control the influenza infection and that novel NA mutations were recognized. Unlike the rapid emergence of resistance to M2 inhibitors, selection of influenza viruses with altered NA usually requires prolonged drug exposure. Based on enzyme inhibition assays, NA mutations frequently confer resistance to a single NAI. The mechanisms of resistance to NAIs are complex and require further investigation. Future studies should assess the impact of HA mutations and sialyation of HA on the virus dependence on the NA activity and NAI susceptibility.

10. Conclusion

Interest in respiratory viruses has greatly expanded over the half year since the Fifth International Symposium on Respiratory Viral Infections largely as the result of the emergence of Severe Acute Respiratory Syndrome (SARS) due to a novel coronavirus. Although SARS was not recognized at the time of the meeting, the symposium succeeded in reviewing recent advances in respiratory virus research. The recent events associated with the SARS epidemic and outbreak of influenza A/H7N7 in Europe further emphasize the importance of planning ahead for pandemics and potential biologic catastrophes. This meeting focused on the importance of pandemic planning for influenza and outlined areas in which vaccine development and implementation needs continued study. The development of live-attenuated and recombinant vaccines holds great promise for preventing viral respiratory diseases in the future. Clearly significant challenges lie ahead in the development of antivirals with activity against the respiratory viruses. In addition to common respiratory viral pathogens like rhinoviruses and RSV, the threats of SARS and novel influenza viruses highlight the medical needs for antivirals for both prevention and treatment.

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