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Review

Current research on respiratory viral infections: Third International Symposium

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1. Introduction

The Third International Symposium on Respiratory Viral Infections was convened by The Macrae Group (New York, NY) in St. Lucia, Windward Islands, on 1–3 December 2000. For the third time, this symposium provided a forum for virologists, vaccinologists, clinicians, pharmacologists and public health specialists to discuss recent advances in respiratory virus research in an interdisciplinary fashion (Kaiser et al., 1999; Munoz et al., 2000). The spectrum of discussion ranged from basic virology and pathogenesis to vaccinology, immunology, and management strategies for respiratory viral infections. Epidemiology of respiratory viral disease and possible preparations for the next influenza pandemic were also an important part of the agenda.

The meeting was chaired by Robert Chanock (NIAID, NIH) and Frederick Hayden (University of Virginia). Invited speakers included Leta Crawford-Miksza (Department of Health, CA), Marshall Horwitz (Albert Einstein College of Medicine), Janet Englund (University of Chicago), Chantelle Ward (Glaxo Wellcome, Stevenage, UK), Peter Collins (NIAID, NIH), John Mills (Macfarlane Burnet Center, Melbourne, Australia), Ruth Karron (Johns Hopkins University), Frank Top (MedImmune, Gaithersburg, MD), Robert Couch (Baylor College of Medicine), Colin Marchant (Boston University), James Crowe (Vanderbilt University), Brian Murphy (NIAID, NIH), Goran Wadell (University Hospital, Umea, Sweden), D.A. Henderson (Johns Hopkins University), Kanta Subbarao (CDC), Gina Kolata (*New York Times*), John O'Connell (Wyeth-Ayerst Research, Pearl River),

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Steve Worland (Agouron Pharmaceuticals, San Diego), Barney Graham (VRC, NIH). Clinical updates on neurminidase inhibitors were presented by Michael Elliott (Glaxo Wellcome), Penelope Ward (Roche) and Frederick Hayden for Cynthia Fowler (R.W. Johnson). Submitted abstracts provided the basis for a poster session and ten additional oral presentations by Eurico Arruda (Sao Paulo University, Brazil), Gregory Gray (Naval Health Research Center, San Diego), Jane Ives (Roche Discovery, Welwyn Garden City, UK), Ron Scott (University of Newcastle, UK), Ruth Tal-Singer (SmithKline Beecham, USA), Albert Osterhaus (Erasmus Medical Center, Rotterdam, The Netherlands), Flor Munoz (Baylor College of Medicine, Houston), Koen Andries (Janssen Research Foundation, Beerse, Belgium), Mark Blatter (Primary Physicians Research, Pittsburgh) and Frederick Hayden (University of Virginia).

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2. Epidemiology and impact

2.1. A historical overview by Robert Chanock

2.1.1. Discovery

Until 1953, influenza viruses were the only known filterable human respiratory tract pathogens. In 1931, Shope recovered an influenza A H1N1 virus from swine, which probably was the first human influenza virus isolated. Two years later Smith, Andrews and Laidlaw recovered the first influenza isolate from humans, and soon after, efforts to develop an inactivated influenza A vaccine began. Influenza B and C were isolated in 1940 and 1947 by Francis and Taylor, respectively. In 1953, Rowe and Huebner, both working in the Laboratory of Infectious Diseases (LID) at the National Institutes of Health, and Hilleman, then working at the Walter Reed Army Medical Center, recovered the first human adenoviruses and established their importance in acute febrile respiratory tract disease (Rowe et al., 1953; Hilleman, 1954). In the following years, Robert Chanock discovered most of the remaining respiratory viruses that are considered important lower respiratory tract pathogens today. The first of them, a croup-associated myxovirus, was discovered during an outbreak of croup in Cincinatti in 1954, and it was later designated human parainfluenza virus type 2 (PIV2). In 1956, Morris and colleagues recovered the chimpanzee coryza agent (CCA) during an outbreak of a cold-like illness in a chimpanzee colony, and a year later Chanock and colleagues recovered two similar isolates from an infant with bronchopneumonia and from another infant with laryngotracheobronchitis, and characterized the human virus now known as respiratory syncytial virus (RSV) (Chanock et al., 1957). The discovery in 1958 of PIV1 and PIV3, the single most common cause of croup and the second most common cause of serious viral pediatric lower respiratory tract disease, respectively, broadened our understanding of the etiology of acute lower respiratory tract disease (Chanock et al., 1958). The discovery of PIV4 in 1960 followed in short order.

In 1961, a double-blind prospective study evaluating the use of tetracycline in the treatment of cold agglutinin-positive atypical pneumonia led to the identification of the etiologic agent of this disease. The agent, originally recovered by Eaton from patients with this form of pneumonia, was known to be filterable and thought to be a virus but its role as an etiologic agent was heavily disputed. A large double-blind study by Chanock, Kingston and Mufson, in which antibodies to the Eaton 'virus' were used to define a subset of patients with pneumonia, showed that tetracycline therapy decreased duration of the disease, thereby excluding a virus etiology. Subsequently, the Eaton agent was shown by Chanock, Hayflick and Barile to be a mycoplasma that grew in cell-free medium; later it was named *Mycoplasma pneumoniae* (Chanock et al., 1962). Serologic analyses and studies in adult volunteers confirmed the etiologic role of this organism in cold agglutinin-positive atypical pneumonia.

2.1.2. Intervention

Renewed efforts in vaccine development against respiratory viruses began in the 1960s with the observation that infants and young children, after having recovered from respiratory tract infection with adenoviruses, shed virus from their gastrointestinal tract for an extended period of time without experiencing gastrointestinal symptoms. This led to the hypothesis that one could potentially use the gastrointestinal tract to vaccinate against respiratory tract disease caused by these viruses. Wild-type adenovirus type 4 and 7 administered orally in enteric-coated capsules was found to protect military recruits against respiratory tract disease caused by these viruses. Gastrointestinal symptoms were not observed, and although virus was shed from the intestine, it did not infect close contacts (Couch et al., 1963).

The development of vaccines against respiratory viruses suffered a major setback in 1966 when formalin-inactivated RSV vaccine not only failed to protect infants against RSV infection but instead potentiated RSV disease upon subsequent RSV infection (Kim et al., 1969). The inactivated vaccine did not induce a potent neutralizing antibody response but it stimulated an exaggerated CD4 + T cell response without stimulating cytotoxic CD8 + T cells. This unanticipated failure of a non-living vaccine reoriented the research agenda of the Laboratory of Infectious Diseases towards the development of live-attenuated virus vaccines. A cold-passaged RSV strain (*cp*52) was selected in 1966 as the first candidate live-attenuated RSV vaccine strain (Friedewald et al., 1968). This candidate vaccine was safe and immunogenic in adults and older children but was insufficiently attenuated in seronegative infants (Kim et al., 1971). Since then, the search for a live RSV vaccine strain has been a central focus of the LID. Developing a live RSV vaccine candidate that is attenuated yet immunogenic in seronegative infants has proved to be a formidable task. Since incidence and morbidity of RSV are highest in the second and third month of life, a vaccine candidate has to be safe for administration to neonates, able to stimulate an immature immune system, and able to overcome the immunosuppressive and antiviral effects of passively acquired maternal RSV antibodies. Initial vaccine candidates were derived in the late 1960s and early 1970s by passage of virus at low temperature (cold passage) or by chemical mutagenesis. Several different lineages of mutants, such as temperature sensitive mutants generated by 5-fluorouracil (5FU) mutagenesis, were evaluated in infants and young children but were insufficiently attenuated or genetically unstable. The cold-passaged (*cp*) mutant that was subsequently further attenuated by the acquisition of two missense mutations that conferred temperature sensitivity (*ts*) to yield *cpts* RSV 248/404, has provided the most promising vaccine candidate tested thus far. This candidate vaccine virus was infectious, safe and immunogenic in 1-month-old seronegative infants, conferred protection against challenge with a second dose of vaccine virus 6 weeks later, and caused only mild upper respiratory tract symptoms (Wright et al., 2000).

Recent development of a method for rescue of infectious RSV from cDNA by Collins enhanced our ability to develop RSV vaccine candidates rapidly (Collins et al., 1995). Site-directed mutagenesis can now be used for the first time to construct viruses with one or more additional attenuating mutations. Using recombinant cDNA technology, viable RSV mutants with deletion of the NS1, NS2, SH or M2-2 gene have been constructed as vaccine candidates that bear genetically stable attenuating mutations. For these reasons, it is likely that a live-attenuated vaccine that exhibits an acceptable balance between attenuation and immunogenicity can be developed within the next several years. This vaccine virus might possess one or more gene deletion mutations together with or without the earlier characterized cold-passaged (*cp*) and temperature-sensitive (*ts*) mutations.

The first PIV vaccine candidates were also prepared by formalin-inactivation. Similar to the experience with formalin-inactivated RSV, these vaccines did not protect against PIV disease. In the early 1980s, Belshe attenuated a PIV3 isolate by 45 passages at 20°C (*cp*45) (Belshe and Hissom, 1982). In clinical studies, the PIV3*cp*45 vaccine candidate has proved to be safe, genetically stable and immunogenic in seronegative in-

fants (Karron et al., 1995). This vaccine candidate is currently being tested in phase II clinical trials. A recombinant version of PIV3*cp*45 has been rescued from cDNA, and the genetic basis of its attenuation (*att*), temperature-sensitivity (*ts*) and cold-adaption (*ca*) phenotypes has been determined (Skiadopoulos et al., 1999).

Influenza A and B virus vaccine development followed this same path of serial passage at 20°C (cold-adaptation) to generate mutants with *att*, *ts* and *ca* phenotypes (Maassab, 1969; Maassab and Bryant, 1999). In contrast to PIV and RSV, live attenuated influenza A vaccine strains were virus reassortants that were generated by mating the attenuated donor virus with an epidemic wild type virus so that the reassortant virus vaccine was a chimera that contained the attenuating genes of the donor virus, while the HA and NA genes were derived from the current epidemic virus (Murphy et al., 1980). This strategy, developed by John Maassab, of using the cold-adapted mutant virus A/AA/6/60 as the donor of the six attenuating internal and non-structural genes for construction of reassortant vaccine strains was validated by the large series of consecutive reassortants that have proven to be attenuated and immunogenic. Analysis of the genetic basis of attenuation showed that the influenza A PB1 and PB2 genes each consistently specified the *ts* phenotype, and PA specified the *ca* phenotype. However, all three of these genes of the viral polymerase complex contribute to the attenuation of the trivalent influenza A (H1N1 and H3N2) and B vaccine viruses (Murphy, 1993; Maassab and Bryant, 1999). The safety, protective efficacy and phenotypic stability were confirmed in large phase III trials, and licensure is expected in the near future (Belshe et al., 1998).

Evidence for the prophylactic effect of serum RSV neutralizing antibodies was demonstrated in the 1980s (Prince et al., 1985b). Passive transfer of homologous RSV convalescent serum to cotton rats protected them against RSV replication in the lungs following subsequent intranasal challenge with wild type virus. A serum RSV neutralizing titer of 1:300 in recipient cotton rats conferred almost complete protection. This amount of neutralizing antibody necessary for protection was later confirmed in clinical trials and today forms the basis for passive RSV prophylaxis in high-risk infants (Groothuis et al., 1993).

2.2. Medical risk factors for severe RSV disease

An increased incidence and severity of RSV disease is seen in preterm infants with or without chronic lung disease (CLD), in children with congenital heart disease (CHD), and in immunosuppressed children and adults. Respiratory disease in general is a common cause for re-hospitalization of preterm infants. Cunningham and colleagues compared a cohort of preterm infants (mean gestational age at birth 28 ± 2 weeks) to a cohort of term infants and found a 10-fold increase in readmission for respiratory disease in preterm infants without CLD, and a 18-fold increase for preterm infants with CLD (2.5, 25 and 45%, respectively) (Cunningham et al., 1991). CLD patients in a home oxygen program were at even higher risk (53% hospitalization, 13% ICU admission) (Groothuis et al., 1988). Children with pulmonary disorders such as cystic fibrosis, lung malformation or recurrent aspiration pneumonitis, when admitted for RSV disease, are as likely to require ICU treatment (13–50%) and mechanical ventilation (up to 33%) as children with CLD (35 and 28%, respectively) (Arnold et al., 1999). Earlier studies of high-risk infants admitted for RSV disease yielded similar results, with ICU treatment necessary in 13–34% and mechanical ventilation necessary in 12–16% (Meert et al., 1990; Navas et al., 1992).

Apart from preterm infants with or without CLD, children with congenital heart disease (CHD) are a second group of high-risk patients, particularly when they suffer from pulmonary hypertension. In a prospective study of 699 children hospitalized in five consecutive RSV seasons (1976–1980), 63% of 27 patients with RSV disease and CHD (compared with 14% of patients without CHD) required ICU treatment and 37% died (MacDonald et al., 1982). In a prospective study of 214 children with CHD, the incidence of RSV-related hospitalization during one RSV season was 15% for children younger than 4 years and 24% for infants younger than 6 months of age (Simoes et al., 1998).

Immunocompromised children are a very diverse population and not all of them are at equal risk for severe RSV infection. Children receiving corticosteroid therapy have a much lower risk for RSV-related hospitalization and death than children receiving chemotherapy for malignancies or children with primary immunodeficiencies. RSV-related mortality was 15% for chemotherapy-recipients and 40% for children with primary immunodeficiencies compared with 0% for steroid-treated children (Hall et al., 1986). RSV grew to very high titer in children receiving chemotherapy, and more than half the patients shed RSV for 3 weeks or longer.

For immunocompromised adults, the picture is not much different from that described for children. Infections are often acquired nosocomially, virus shedding is prolonged, and the incidence of pneumonia and death is high. RSV is the most important viral respiratory pathogen in these patients, followed by picornaviruses, influenza and parainfluenza viruses: 60% of confirmed RSV infections in leukemia and bone marrow transplant patients resulted in pneumonia, and the fatality rate was greater than 30% (Couch et al., 1997). Whimbey and colleagues reported RSV case fatality rates for bone marrow transplant recipients as high as 31% when therapy was administered early and adequately (ribavirin and IVIG), and 100%, when therapy was initiated late or inadequately (Whimbey et al., 1997).

HIV-infected children in an urban setting in South Africa also have an increased burden of viral lower respiratory tract illness (LRI) although respiratory viruses are less frequently isolated from nasopharyngeal aspirates of HIV-infected children than from children without HIV infection. The relative risk for severe LRI caused by RSV was twice as high in HIV-infected than in uninfected children two years of age or younger (Madhi et al., 2000).

2.3. Impact of RSV in a native American population

Little information is available regarding genetic and environmental factors in susceptibility to RSV infections. Most RSV epidemiologic studies are conducted in affluent countries and temperate climates although RSV is thought to be the leading cause of severe viral acute respiratory infections (ARI) in infants around the globe (Weber et al., 1998). A 3-year prospective surveillance study was conducted in the Yukon–Kuskokwim (YK) Delta of South-Western Alaska to determine the rate and severity of RSV infections requiring hospitalization for infants in this Yupik Eskimo population (Karron et al., 1999). The annual rate of RSV hospitalization for YK delta infants less than 1 year of age was unusually high, i.e. 53–249/1000. One in 125 children born in the YK Delta, compared with between one in 550 to one in 11 000 infants in affluent countries (Sims et al., 1976; Martin et al., 1978; Glezen et al., 1981), required ventilatory support for RSV disease. RSV infection was the single most frequent cause of hospitalization of YK delta infants. As in temperate climates, RSV epidemics in the YK Delta occur annually from November through June, with peak hospitalizations for RSV disease occurring between November and February. Within sub-regions of the YK Delta, epidemics were as brief as 1 month, probably because there is very limited traffic between villages in the winter months. Most of the infants admitted to hospital were less than 1 year of age and had no medical risk factors for RSV disease. Surprisingly, 9% of the admitted infants in the YK delta and 8% of infants in a comparison group admitted to Johns Hopkins Hospital (JHH) were less than one month old. Of children with severe disease, 34% in the YK delta and 24% at JHH were less than 2 months old. Disease severity in non-high risk children did not differ between children admitted to JHH or YK Delta regional hospital, suggesting that differences in hospitalization practices could not account for the high rates of hospitalization for RSV in YK Delta infants. In the YK Delta, 19% of those admitted for RSV disease were readmitted within a single RSV season. Severity of RSV disease, age at first illness and receipt of ribavirin were all associated with readmission. In infants less than 6 months of age, a low neutralizing antibody titer in cord blood samples was strongly associated with severe disease.

A questionnaire-based case control study that was matched for age and sub-region in the YK delta detected three risk factors influencing RSV hospital admission. Medical risk factors (prematurity, chronic lung disease, congenital heart disease) increased the risk of admission 6.25-fold. More than eight people living in one household doubled the risk of hospitalization while breastfeeding had a protective effect. Smoking, food-pre chewing and economic status were not significantly associated with RSV hospitalization.

This study may be useful in the continued analysis of the impact of RSV in developing countries. As in the YK delta, RSV is the leading cause of viral lower respiratory tract disease in most developing countries, but lack of access to diagnostic reagents and hospital facilities has made it difficult to quantify the impact of RSV. Also, the rate of severe RSV disease in term neonates was much higher than in earlier studies, both in the YK Delta population and the comparison group in Baltimore. These findings should be confirmed in other populations because they have important implications for RSV vaccine development.

2.4. Respiratory syncytial virus (RSV) and human rhinovirus (HRV) infections in children with AIDS and lower respiratory tract illness (LRTI)

Acute respiratory infections (ARI) cause a great burden of disease in developing countries (de Arruda et al., 1991). Although a growing number of children from developing nations are HIV infected, there is little knowledge about the frequency and severity of viral ARI in HIV infected children. Earlier studies of viral pathogens in immunocompromised adults indicated that CMV, herpes simplex, influenza, parainfluenza, rhinovirus, adenovirus, enterovirus, and RSV cause lower respiratory infection (Connolly et al., 1994). A recent study assessed the frequency of RSV and rhinoviruses (HRV) in hospitalized children with or without AIDS, who presented with lower respiratory tract infection (LRTI). About 73 episodes of LRTI in children with AIDS and 73 in children without HIV infection, matched by age and sample collection month, were studied in a rural area of southern Brazil. The frequency of RSV infection was highest in the fall and winter, between February and July, whereas HRV was detected throughout the year. RSV was found in 8/73 (11%) and 9/73 (12%) of LRTI episodes in AIDS and non-HIV infected children, respectively. HRV was found in 16/73 (22%) and 12/73 (16%) of the episodes in children with AIDS and HIV-uninfected children, respectively. No difference was detected in frequencies of HRV and RSV infections between the two groups. HRV infections, however, tended to be more frequently associated with pneumonia in children with AIDS (7/16, 44%) than in the control group (1/12, 8%) ($P = 0.09$). Other clinical presentations of LRTI were observed with equal frequency. These findings did not confirm HRV as a causative agent of pneumonia in children with AIDS, but suggest that further studies of LRTI are desirable and that interventions for HRV could be considered for immunocompromised children with LRTI.

2.5. Adenovirus: old and new syndromes

The human adenoviruses (Ads) are a large family of over 50 serotypes, as well as numerous variants and intermediates. They are divided into six subgroups (A–F) that exhibit different tissue tropism. The clinical manifestations of adenoviral disease are protean. The most common are respiratory syndromes in both children and adults. It is estimated that Ads cause 5–15% of all respiratory disease in children, including pharyngitis, tonsillitis and pertussis-like syndrome. In children and adults, Ads are also associated with lower respiratory tract infections such as bronchitis and pneumonia. Subgroup D Ads are associated with both epidemic and sporadic ocular infections including conjunctivitis and keratoconjunctivitis. Due to their stability in the environment, these viruses are highly transmissible, particularly in nosocomial settings. In adults, Ads cause large-scale epidemics of acute respiratory disease (ARD) in closed populations of military recruits, dormitory residents and long-term care facility occupants, which are primarily associated with Ad serotypes 4 and 7a, and to a lesser extent with serotypes 2, 3, 11, 21 and 35. While replication in the gastrointestinal tract is a feature of most Ad

infections, only Ad40 and Ad41 (subgroup F) are associated with gastroenteritis in infants and young children.

Ad infections in immunocompromised subjects are increasing as their numbers increase, with severe consequences. Clinical manifestations of Ad infection in immunocompromised patients include pneumonia, hepatitis, encephalitis, and systemic and disseminated disease, with case fatality rates from 18 to 60%, depending on the nature of the immunodeficiency. Nearly all Ad serotypes have been associated with these infections, but the higher numbered subgroup D serotypes that are usually not associated with clinical disease in the immunocompetent host have been especially common.

A number of 'new' disease associations with Ad infection have recently been reported, probably due to improved highly sensitive molecular diagnostic techniques that also increase the probability of laboratory contamination. Detection of viral genome in the absence of positive viral culture has been described in cases of myocarditis and pericarditis in children and adults (Martin et al., 1994; Bowles et al., 1999; Pauschinger et al., 1999), sudden infant death (Shimizu et al., 1995), toxic shock-like syndrome (Price, 2000) and 'unexplained death' (Perkins et al., 1996). Isolation of Ads from patients with central nervous system manifestations of fatal acute flaccid paralysis (Cardosa et al., 1999) and encephalitis with cerebral edema (Chatterjee et al., 2000) has also been reported.

It is, perhaps, not surprising that the clinical manifestations of Ad infection are evolving because the viruses themselves are in the process of continuous evolution. The Ad mutational repertoire includes homologous recombination, illegitimate recombination, and single base mutation (SBM). Homologous recombination occurs in conserved regions of the genome between closely related viruses within the same subgroup, and it requires regions of homology in the two parent strands. It is the primary mechanism responsible for intermediate Ads, which are mosaic viruses with shared hexon characteristics, or with the hexon characteristics of one type and fiber of another. Illegitimate recombination requires only short regions of homology of one to three nucleotides (short direct repeats), and is thought to be the result of polymerase stuttering or slippage. It causes deletions, insertions and duplications of short regions of DNA. In Ads it occurs in noncoding regions and hypervariable regions (HVRs) of hexon capsid proteins that tolerate structural variation. The HVRs of the hexon contain the viral neutralization epitopes, so that mutations in these regions result in deletion, formation or alteration of these epitopes, leading to antigenic shift. It is the primary mechanism by which new serotypes arise, particularly among the fastest growing group, the subgroup D Ads. Single base mutations accumulate gradually across the viral genome but can occur at a 30-fold higher rate in the HVRs, where they cause incremental antigenic drift and the creation of variant strains. Ad evolution is compounded by all three mechanisms and perhaps others that have yet to be defined. As a result, serological identification of subgroup B2 and D Ads has become extremely difficult. It seems reasonable that the time has come to consider a sequence-based Ad classification system, similar to that in use for papillomaviruses and enteroviruses.

2.6. Adult adenovirus infection: loss of orphan vaccine precipitates US military respiratory disease epidemics

Adenovirus has re-emerged as a leading cause of febrile respiratory disease among military recruits. Large and frequent epidemics were common at trainee camps before 1971 but were eliminated with the introduction of the live enteric type 4 and 7 vaccines. In 1996, the sole vaccine manufacturer discontinued production of these live enteric coated vaccines because of contractual issues. While limited vaccine stores were still available in 1997 and 1998, vaccine stores were completely depleted by early 1999. To monitor the effect of discontinued vaccination, weekly surveillance for febrile respiratory infections (FRI), defined as oral temperature >100.5°F with respiratory disease symptoms, was conducted from October 1996–June 1998 at four military training camps. During this interval, 1814 (53%) of 3413

throat cultures yielded adenovirus. During the winter of 1997–1998, adenovirus infections caused more than 90% of FRI at each of the four camps. Ad 4, 7, 3, and 21 accounted for 57, 25, 9, and 7% of the isolates, respectively. Three training camps experienced a high prevalence of adenovirus type 4 and the fourth camp experienced a type 7 outbreak. Among symptomatic trainees, those who did not receive vaccine were 28 times more likely to be infected by Ad 4 or 7 than vaccinated subjects (Gray et al., 2000).

Surveillance was extended to eight sites in June 1998 and virus isolation was attempted for adenovirus, influenza A and B, RSV, and parainfluenza 1–3. Large Ad 4 epidemics were observed in six training camps throughout the US, while RSV and influenza A and B viruses were isolated less frequently. The impact of adenovirus epidemics on basic training can hardly be overestimated. Recruit camps were forced to convert barracks into special infirmaries to care for the ill, and hospitals were forced to halt elective surgeries. At one camp, the number of trainees that had to repeat their basic training because of extended illness increased 20-fold. This 'recycling' has an extremely negative effect on the morale of trainees and it impacts on the military's readiness. As many as 1800 preventable adenoviral trainee medical encounters occurred during the winter months of 1999 and in 2000; two military trainees died with molecular evidence of acute adenoviral infection, one with encephalomyelitis and another with acute respiratory distress syndrome (ARDS). Vaccination against Ad 4 and 7 has proven to be extremely safe and effective, and to prevent an enormous burden of disease in military trainees (Howell et al., 1998). It is urgent that a new manufacturer for adenoviral vaccines be identified, and vaccine production must resume as soon as possible.

3. Virology and detection

3.1. Adenovirus: new insights

In recent years, much progress has been made in understanding virus-induced modulations of the host immune response to viral infections. For Ad, more than 20 viral gene products are known to participate in the modulation of immune responses, and many of these gene products are expressed from genes clustered in the early region 3 (E3) (Wold et al., 1999; Horwitz, 2001). The overall effect of Ad E3 gene products on immune responses in vivo can be appreciated from the results of three studies in mice that investigated transplant rejection and development of autoimmune disease. In the first study, the expression of the complete Ad E3 cassette in pancreatic islet cells as transgenes under the control of the rat insulin promoter (RIP) enabled allogeneic islet donor cells containing the H-2^{bxd} class I MHC to be accepted long-term by H-2^{d} recipient mice (Efrat et al., 1995), indicating that Ad E3 gene products could potentially be used as a powerful tool in the control of transplant rejection. The second study used the lymphocytic choriomeningitis virus (LCMV) model of autoimmune diabetes mellitus, in which the LCMV proteins NP or GP are expressed on the surface of islet cells, and diabetes is induced by infection with LCMV that induces CD8 + (GP) or CD8 + and CD4 + (NP) T-cell mediated immune responses. In this model, the co-expression of RIP-E3 with LCMV-NP or GP completely prevented the onset of diabetes after LCMV infection (von Herrath et al., 1997). Similar protective effects of Ad E3 transgenes were seen in a third study that used the non-obese diabetic mice (NOD) model of diabetes mellitus, and the underlying mechanisms are currently being investigated (Efrat et al., 2001). Thus, as the understanding of the mechanism of action of the Ad E3 immunoregulatory genes are being pursued in various systems, they are being utilized to control selected immune reactions that might be involved in the genesis of autoimmune diabetes.

Some of the better-characterized gene products of the E3 region are (in order of increasing distance from the E3 promoter) gp19, 11.6, 10.4, 14.5 and 14.7k. Only the functions of gp19 and 14.7k shall be discussed here; the other Ad E3 gene products have been reviewed elsewhere (Horwitz, 2001). In vitro, gp19k reduces the expression of class I major histocompatibility complex

(MHC) molecules by retaining the MHC heavy chain in the endoplasmic reticulum or retrieving it back from the Golgi, and also by inhibiting peptide processing (Bennett et al., 1999). In the cotton rat model of adenovirus pneumonia, gp19k deletion mutants replicate like wild-type virus but they induce a much stronger inflammatory response (Ginsberg et al., 1989), whereas in C57BL mice an increase in pulmonary pathology is not seen (Sparer et al., 1996).

The Ad E3 14.7k protein inhibits TNFα-induced cell death by a process that does not involve down-regulation of the TNFα-receptor. In cotton rats (Ginsberg et al., 1989) and C57BL mice (Sparer et al., 1996) deletions of Ad E3 14.7K modify the pulmonary inflammatory response, i.e. an increase of polymorphonuclear leukocytes in cotton rats and more pronounced alveolar infiltration in mice. In order to determine how the Ad 14.7K protein prevents cell death, the cell proteins that interact with this viral protein were determined. Using a yeast two-hybrid system, four 14.7k-interacting proteins (FIPs) were identified. Three of them have been characterized and have been shown to participate in quite diverse cellular pathways (Li et al., 1997, 1998, 1999b). FIP-1 (also known as Rag-A, a Ras-related small GTPase) can bind to TCTEL, a component of the microtubule motor protein dynein, forming 14.7k-FIP-1-TCTEL complexes (Lukashok et al., 2000), and 14.7k has been postulated to affect microtubule dependent macromolecular transport or even modulate the transport of virus. However, because 14.7k is not a structural protein of adenoviruses and must be made de novo from early viral transcripts, it is unlikely to play a role during viral entry, even though the process is known to be microtubule dependent. The role of 14.7k during viral exit from cells has not been studied. FIP-1 also binds to a second GTPase (GIP-2) that localizes in the centrosome and in addition to potential effects during mitosis may be involved in transporting macromolecules between the nucleus and the cytoplasm. FIP-2 binds to abnormal huntingtin, and more specifically to the expanded polyglutamine tract that appears to be associated with cell death of neurons in Huntington's disease (Faber et al., 1998). Whether or not Ad E3-14.7k and/or FIP-2 can prevent Huntingtin-induced cell death is currently being investigated. Over-expression of FIP-3, which is also called NF-κB essential modulator (NEMO) or inhibitor of kappa kinase gamma (IKKγ) causes morphologic changes and eventually apoptosis in a variety of cell lines. The amount of apoptosis induced by FIP-3 can be reduced by 70% when Ad E3-14.7k is present. Apart from 14.7k, FIP-3 seems to interact with a number of key molecules in the TNF receptor and NF-κB signaling pathways such as the receptor interacting protein (RIP), the inhibitor of kappa B kinase beta (IKKβ) and the NF-κB inducing kinase (NIK) (Li et al., 1999b). These few examples of the effects Ad E3 gene products have on the pathobiology of diseases as different as autoimmune diabetes, transplant rejection and Huntington's disease indicate how much remains to be learned from studying adenovirus-host interactions.

3.2. Virulence factors in RSV: application of reverse genetics

The RSV (strain A2) genome is a single stranded negative-sense RNA of 15 222 nucleotides that is transcribed into 11 major subgenomic mRNAs. Three of the eleven encoded proteins are transmembrane proteins. The G protein mediates attachment to cell surface receptors, the F protein mediates virus–cell and cell–cell fusion, and the function of the SH protein is unknown. Other structural proteins are the M protein, which plays a role in virion assembly, the N, P and L proteins that make up the viral polymerase, and the M2 ORF1 protein that functions as a transcription anti-termination factor. The other proteins include two non-structural species, NS1 and NS2, and the M2-2 protein encoded by the second ORF of the M2 mRNA. Using a reverse genetics system to rescue infectious RSV from cDNA, five of the 11 genes of RSV can be ablated individually and in some cases in combination without rendering the virus non-viable (Collins et al., 1999; Jin et al., 2000b). These five non-essential genes are NS1, NS2, SH, G, and M2-2. Since all of these genes confer a selective

advantage to RSV in vitro and/or in vivo, they can be described as virulence factors — the deletion of which will lead to attenuation of the virus. Deletion of the small hydrophobic (SH) transmembrane protein yields a recombinant RSV called RSVA2ΔSH, which replicates in vitro as well as wild type (wt) RSV and induces plaques in HEp-2 cells that are larger than wt RSV plaques. There is no reduction in synthesis of RNA or protein associated with the deletion of SH. In chimpanzees, however, the virus is slightly attenuated (Whitehead et al., 1999a). Deletion of the NS1 or NS2 gene results in a substantial reduction in replicative efficacy in vitro, and this reduction is more pronounced in HEp-2 cells than in Vero cells (which lack interferon α and β genes), suggesting that these two genes act as antagonists to type 1 interferon effects. Deletion of NS1 and NS2 from bovine RSV provided direct evidence that NS1 and NS2 cooperatively antagonize α/β interferon-induced antiviral responses (Schlender et al. 2000). In chimpanzees, the level of replication of both RSVA2ΔNS1 and RSVA2ΔNS2 is reduced greater than 10 000-fold in the lower respiratory tract. In the upper respiratory tract, the ΔNS1 virus is more attenuated than the ΔNS2 virus (Teng et al., 2000). Deletion of the M2 ORF2 not only identifies a markedly attenuated RSV mutant but reveals an important role for this ORF in the replicative cycle of RSV (Bermingham and Collins, 1999). During infection with wild-type RSV, transcription appears to shut off at approximately 12–16 h post infection while RNA replication increases concurrently. In contrast, this apparent switch from transcription to RNA replication was not observed for the RSVΔM2-2 virus, implying that M2-2 is a regulatory protein involved in the shift. Instead, transcription continued to increase while RNA replication remained low compared to wild-type RSV. Overall, gene expression was increased 7–18 fold. The synthesis of the G and F proteins also was increased and resulted in increased syncytium formation. Replication of the RSVΔM2-2 virus in vitro was attenuated, probably due to reduced RNA replication (Bermingham and Collins, 1999; Jin et al., 2000a). In chimpanzees, comparison of the four RSV gene deletion mutants mentioned above with wt RSV and the incompletely attenuated RSV *cpts*-248/404 vaccine candidate results in a hierarchy of increasingly more attenuated viruses — wt RSV < ΔSH < ΔNS2 < 248/404 < ΔNS1 < ΔM2-2. The final RSV gene deletion mutant, RSVA2ΔG, was not evaluated in chimpanzees because the absence of this major protective antigen would not be desirable in a vaccine virus. RSVA2ΔG replicates as efficiently as wt RSV in Vero cells, showing that G is not essential for efficient virus replication. However, the RSVΔG virus is highly attenuated in Balb/c mice, indicating the importance of the RSV G protein in vivo. It is evident from the above ranking that RSV reverse genetics is able to generate mutants exhibiting gradations in their level in attenuation (Collins et al. 1999). This menu of viruses with different levels of attenuation is crucial in identifying an RSV vaccine that exhibits the desired balance between attenuation and immunogenicity in seronegative infants. Since clinical data indicate that RSV 248/404 is just slightly under-attenuated in the 1-month-old target population, the ΔNS1 mutant could be exactly what is needed.

3.3. Protein–protein interactions in RSV assembly

In order for RSV assembly to be an efficient process, viral structural proteins must be brought together in a coordinated fashion (Peeples, 1991; Lenard, 1996). Compared with other paramyxoviruses RSV exhibits several unique features. The G, M2-1 and M2-2 proteins are found only in the *Pneumovirus* genus of the *Paramyxoviridae*, and the role these proteins play in RSV assembly is much less well understood than the role of the F, HN and M proteins of other *Paramyxoviridae* (Collins et al., 1996). Comparison of multiple human, bovine and ovine RSV strains shows that the cytoplasmic domains of the F and G proteins are well conserved amongst human RSV strains and subtypes, and conserved to some degree between the three species. In analogy to other paramyxoviruses, it is likely that the cytoplasmic domain of F and G interact in the process of virion assembly with cellular proteins involved in protein trafficking and in the polarized budding

process, as well as with other viral proteins. The RSV G and M proteins co-localize in the Golgi apparatus, not only in RSV-infected cells but also in cells transfected with only the G and M proteins, indicating that other viral proteins are not needed for this interaction (Peroulis et al., 2001). The G–M interaction is seen with full-length G protein but not with a secreted form of G that lacks the conserved cytoplasmic domain and transmembrane domains. Systematic deletion and substitution mutagenesis of the cytoplasmic domain of G has identified a sequence-specific, six amino acid motif that directly interacts with M (Peroulis et al., 2001).

During RSV infection M protein can initially be detected in the nucleus, but later in the infectious cycle it is found in inclusions within the cytoplasm (Ghildyal et al., unpublished). The N and P proteins of RSV were earlier shown to be necessary and sufficient for these inclusions to form, and the RSV M2-1 and L proteins were also shown to be present in these inclusions (Garcia et al., 1993). These same investigators were unable to identify M protein in the inclusions (Garcia et al., 1993). Using confocal immunofluorescent microscopy of infected and co-transfected cells the M protein was shown to be present in these inclusions (Ghildyal et al., 2001). M protein does not localize to the inclusions unless M2-1 is also present (Ghildyal et al., 2001). The M and M2-1 proteins not only co-localize by confocal microscopy but also interact in a protein overlay assay (Ghildyal et al., unpublished).

Taken together, these data suggest that, as with other single stranded negative-sense viruses (Peeples, 1991; Lenard, 1996), the RSV M protein seems to play a crucial role in RSV assembly; bringing the nucleocapsid together with the envelope proteins by binding to the cytoplasmic domains of G and F and to the nucleocapsid proteins N and P together with or via M2-1. The interaction between M, M2-1 and the nucleocapsid proteins might be more complex than outlined here, and might involve additional cellular or viral proteins. To better understand RSV assembly, the domains in the M protein that interact with G, F and M2-1 will have to be defined.

3.4. Reduction in the ability of influenza A virus carrying the R292K mutation to transmit has been demonstrated in ferrets

While influenza viruses readily develop resistance to older antivirals such as amantadine or rimantadine, resistance to neuraminidase inhibitors occurs much less frequently. Nonetheless, resistant influenza A virus mutants can be isolated from patients treated with oseltamivir (Treanor et al., 2000). One of the resistant mutants carries an arginine to lysine mutation at position 292 (R292K) of the neuraminidase protein, causing a reduction in substrate binding and enzymatic activity, as well as resistance to oseltamivir (McKimm-Breschkin, 2000). The infectivity of influenza A viruses carrying the R292K mutation was earlier found to be markedly reduced in mice and ferrets. Transmission of an influenza A H3N2 clinical isolate with a R292K mutation was studied in ferrets in comparison to transmission of the parent wt H3N2 virus that was isolated from the same patient. Donor ferrets (four per group) were inoculated intranasally with the R292K mutant or wt virus and housed with three naïve contact ferrets per donor ferret. The four donors inoculated with wt virus were infected and transmitted virus to each of the 12 contacts. Wild type virus replicated to between 10^4 and 10^5 pfu/ml nasal aspirate. Only two of four donor ferrets inoculated with mutant virus became infected, and the level of replication of mutant virus was reduced 10–100-fold compared with that of wt virus. However, both infected ferrets transmitted virus to contacts. One of them transmitted the R292K virus to one of three contacts only, with virus detected at very low titer on 1 day only. The other donor transmitted virus to all three contacts, and the transmitted virus replicated to titers greater than 10^4 pfu/ml in the contact ferrets. Sequence analysis showed that the donor virus was a mixed population of mutant and wt virus and that only wt virus was recovered from contacts. These data confirm the reduced infectivity of oseltamivir resistant R292K mutants. Effective transmission of the R292K mutant virus in ferrets was not observed, suggesting that the transmission of oseltamivir-resistant virus from human to human

will be unlikely, even during widespread use of NA inhibitors in the treatment of influenza.

3.5. Respiratory virus diagnosis in immunocompromised patients and the elderly

Respiratory virus infections in immunocompromised patients are characterized by persistence of viral infection, prolonged shedding of virus, a high rate of nosocomial acquisition, and a high frequency of pneumonia and death. Similarly, respiratory virus infections in the elderly are responsible for a substantial amount of morbidity and mortality. Detection of respiratory virus infections in these high-risk patients is important for several reasons. It enables the initiation of specific isolation procedures, initiation of specific antiviral therapy, cessation of unnecessary therapy, tests and procedures, and it can aid in identifying and preventing potential outbreaks. Respiratory virus infections can be diagnosed using serology and culture techniques, as well as newer methods such as antigen-detection by enzyme-linked immunoassay (EIA) or immunofluorescence (IFA), enzymatic detection by chemical reactions, or amplification of parts of the viral genome (PCR). Serology is not useful in the acute phase of most illnesses and is of limited usefulness in immunocompromised patients, the elderly or those receiving blood products. Recovery of virus in cell culture is still seen as the gold standard by many but the importance of obtaining a good specimen to ensure that the culture is not falsely negative is often overlooked. A good clinical specimen is the most critical factor in ensuring a correct diagnosis regardless of the diagnostic method used, although it is perhaps most important for culture. Use of nasal wash to obtain a specimen for virus isolation is well-tolerated in cooperative adults and, compared to nasal swabs or throat swabs, increases the sensitivity of cell culture for virus detection. Other factors critical to laboratory success are the use of appropriate transport media, temperature of transport/incubation and time until processing (Atmar and Englund, 1997). This is particularly important in the case of RSV infections in immunocompromised or elderly patients, who often have a relatively low viral titer such as 10^2–10^3 pfu/ml, whereas children often have a titer greater than 10^6 pfu/ml of nasal wash or bronchoalveolar lavage fluid (Englund et al., 1996).

For RSV, parainfluenza and influenza virus there are a number of commercially available rapid detection kits. Multiple simultaneous RT-PCR for detection of RSV, influenza A and B, and PIV1, 2 and 3 (Hexaplex®) was tested in 763 pediatric samples and yielded 100% sensitivity and 98% specificity as compared with culture (Fan et al., 1998), with only 8 h processing time. In a separate study, PCR for respiratory viruses in adult patients with hematologic malignancies was found to be as sensitive as culture (van Kraaj and van Elden, 2000). For influenza, several antigen detection kits ((Directigen, FluOIA and Quick-Vue) and one neuraminidase (ZstatFlu) assay are available to detect virus, with sensitivities ranging from 50 to 96% and specificities ranging from 52 to 99%. Rapid diagnosis of influenza in pediatric patients leads to a decrease in the frequency and duration of antibiotic use and an increase in the frequency of antiviral therapy (Noyola and Demmler, 2000). Rapid diagnosis kits for the detection of RSV in children range in sensitivity from 61 to 87% and in specificity from 93 to 97%, with some test kits performing better than others (Dominguez et al., 1993). In immunocompromised adults, however, the sensitivity of antigen detection kits from nasal wash or throat swab sample was only 15%, while endotracheal or bronchoalveolar sampling increased sensitivity to 70 or 80%, respectively, (Englund et al., 1996).

3.6. Real time PCR: quantifying viral load

Several studies in recent years have highlighted the importance of upper respiratory tract infections in the exacerbation of asthma in children. Freymuth and colleagues reported human rhinovirus (HRV) (46.9%) and RSV (21.2%) as the most frequent pathogens detected in patients with exacerbation of asthma, and noted that PCR increased detection rates 5.8- and 1.6-fold in HRV and RSV infections, respectively, over that of conventional assays (Freymuth et al., 1999). In a study of wheezing children between 2 months and

16 years of age, respiratory viruses were detected in 82%, with RSV in infants (detected in 68% of subjects) and HRV in older children (71%) as the predominant pathogens. Both were strongly associated with wheezing (Rakes et al., 1999). In a community-based longitudinal study, respiratory viruses were detected in 80% of episodes of acute illness with reduced peak expiratory flow, 80% of episodes of wheezing, and in 85% of episodes of upper respiratory symptoms, cough, wheezing, and a fall in peak expiratory flow (Johnston et al., 1995). In these settings, RT-PCR provides a fast and sensitive method to detect RNA viruses. Real time quantitative PCR assays can be similar to conventional PCR in specificity and speed. However, real-time PCR can also quantify viral load (quantity of virus in respiratory secretions) during asthma exacerbations, and it is sometimes more sensitive than conventional PCR. Real time TaqMan quantitative PCR allows estimation of the input viral genome copy number by including a fluorescence reporter on one end and a quenching molecule on the other. The reporter does not fluoresce until the quencher has been cleaved off by the exonuclease activity of Taq polymerase, permitting an estimate of the quantity of PCR product. Fluorescence is being measured continuously every seven seconds, and quantification of the target is based on the number of PCR cycles it takes to produce detectable fluorescence. A retrospective study was conducted in 117 asthmatic children 9–11 years old to compare viral loads in quiescent and exacerbation periods of asthma using the TaqMan technology. Nasal aspirates had been collected earlier and records of peak flow measurement and clinical scoring were available. A quiescent period was defined as absence of clinical symptoms for two weeks or longer. Real time quantitative PCR detected respiratory viruses (mostly rhinoviruses) in 97% of the children with an asthma exacerbation and in 42% of children in quiescence. Viral load was higher in children with exacerbation of asthma than in those with quiescent asthma and higher viral loads also correlated with more severe clinical disease. Although the study showed that real time quantitative PCR is more sensitive than nonnested conventional PCR and also allowed estimation of viral load, there is the caveat that the study had a retrospective design.

4. Pathogenesis and immunology

4.1. Immune determinants of resistance to acute viral respiratory tract disease

Modalities of immunity to acute viral respiratory infections are both specific and nonspecific, humoral and cell mediated. Fever, interferon (IFN), tumor necrosis factor (TNF), natural killer cells and activated macrophages are non-specific modalities stimulated by infection that are capable of mediating antiviral effects. Lung collectins such as Sp-A, have also been implicated in the control of viral respiratory infections (Ghildyal et al., 1999). Specific modalities are antibody in serum and secretions, lymphocyte proliferation responses with cytokine release, and cytotoxic lymphocytes (CTLs). All modalities participate, to some degree, in containing an infection and promoting recovery either via inactivation of free virus or elimination of infected cells. Thus, there is considerable redundancy in the mechanisms controlling the virus infection and promoting recovery. On the other hand, protection against infection is primarily conveyed by specific antibody. Sufficient data is available to conclude that serum IgG neutralizing antibody is the primary mediator of resistance to influenza virus infection, presumably because infection is initiated in the lower respiratory tract and IgG antibody derived from serum is the dominant antibody isotype at that site (Couch and Kasel, 1983). In contrast, IgA antibody is the primary mediator of resistance to rhinovirus and coronavirus infection because evidence indicates these infections are initiated in the nasopharynx where IgA is the dominant antibody isotype (Cate et al., 1966). Since primary infections with influenza virus, RSV, or PIV induce disease in both the upper and lower respiratory tract, both IgG and IgA antibodies are correlates of immunity to infection and disease (Crowe, 1999). Although adenoviruses also cause upper and lower respiratory disease, only serum antibody has been shown to correlate

with immunity. Reinfection with homologous RSV, PIV, rhinovirus and coronavirus can occur but is generally confined to the upper respiratory tract, presumably because IgG antibody in protective quantities is more durable in serum (and lower respiratory secretions) than is IgA antibody in nasopharyngeal secretions. For RSV, the risk of infection declined from 74% for naïve subjects to 56 or 33% after one or two infections, respectively; the risk of lower respiratory disease in these groups declined from 18 to 15 to 3%, respectively, (Glezen et al., 1986). For a rhinovirus, resistance to reinfection of the nasopharynx correlated with the titer of IgA antibody in secretions (Cate et al., 1966).

Rodent models of viral respiratory disease provided much of the early data on immune modalities conferring protection (Crowe, 1999). Both CD4+ and CD8+ T cells can effect clearance of influenza A virus, RSV and PIV in mice in the absence of the other cell type (Lightman et al., 1987). While CD8+ cytotoxic lymphocytes (CTL) mediate immunity through lysis of infected cells and expression of antiviral cytokines, CD4+ T cells exhibit limited direct antiviral activity but play a role in activating B cells and in inducing antiviral cytokine expression (Epstein et al., 1998). There is a general consensus that CTLs contribute significantly to the resolution of primary infections with influenza, RSV and PIV in rodents.

While the correlation between antibody response and protection in humans was established decades ago, cellular immune responses were studied much later, and understanding of the development of these responses in infants and immunosenescence of them in the elderly is still incomplete. Whether or not CTLs in humans convey immunity to respiratory virus infection in the lower respiratory tract and whether they promote clearance of virus in the upper respiratory tract of humans remains to be elucidated. However, for RSV disease in humans, a correlation has been observed between the presence of RSV-specific CTLs in year 1 and absence of severe RSV disease in year 2 (Mbawuike, in press). Morever, the level of influenza-specific CTLs correlated inversely with the quantity of virus in nasal secretions after challenge of humans, and the ability of CTLs to function at this site was demonstrated by a reduction in influenza virus titer in nasal turbinates of mice which were adoptively immunized with purified CD8+ CTLs before intranasal challenge (McMichael et al., 1983 Mbawuike, personal communication).

4.2. Virus-bacterial interactions in the respiratory tract

Knowledge regarding viral-bacterial interactions in the respiratory tract goes back at least to the 1918 influenza pandemic, and interactions have been described for later influenza pandemics as well. Epidemics and pandemics of influenza have been followed regularly by an increase in the incidence of bacterial pneumonia (Schwarzmann et al., 1971; Cartwright et al., 1991). Associations between RSV and *Haemophilus influenzae*, *Bordetella pertussis*, *Neisseria meningitidis* and *Staphylococcus aureus* infections have also been described (Patel et al., 1992; Jiang et al., 1999). Interactions between viral and bacterial diseases are fairly complex and the underlying mechanisms are only beginning to be elucidated. It is known that up-regulation of TNFα and IL-1 increases adherence and uptake of pneumococci (Cundell et al., 1995), and that several cellular receptors for bacterial adherence are up-regulated by viral infections. *S. pneumoniae* and *H. influenzae* interact with PAF receptors (Swords et al., 2000), and *N. meningitidis* interacts with CD14 and CD18 (Raza et al., 1999). Not all interactions are regulated at the level of cell surface receptors. In the mouse influenza model, for instance, severe damage and desquamation of the respiratory epithelium enables access of *S. pneumonia* to the basal membrane and thereby increases the risk for invasive disease (Plotkowski et al., 1986).

Otitis media was traditionally thought to be a purely bacterial infection, with *S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* as the main pathogens. More recent studies, however, indicate that the majority of acute otitis media (AOM) cases are a result of mixed bacterial and viral infection. Heikkinen and colleagues reported an

increase of RSV, parainfluenza virus, influenza or adenovirus infection in children with otitis media (Heikkinen et al., 1999). All respiratory viral infections of the nasopharynx are thought to predispose to AOM but some viruses, e.g. RSV, are frequently found in the middle ear during AOM. The mechanism underlying this respiratory tract infection-AOM sequence involves eustachian tube obstruction, leading to negative middle ear pressure and inspissation of bacteria into the middle ear (Giebink et al., 1980). Children with RSV, adenovirus or influenza virus infections have a 30% risk of developing AOM within 2 weeks of the onset of the respiratory tract infection (Henderson et al., 1982), and coinfection with bacteria and viruses also adversely influences the outcome of AOM. If AOM does not respond to antibiotic therapy within 48 h, it is more likely to involve a viral infection (Arola et al., 1990). The effects of viral co-infection complicate the evaluation of clinical efficacy of anti-bacterial drugs in the treatment of AOM (Marchant et al., 1992). With bacterial-viral co-infection in AOM, there is also delayed clearance of bacteria from the middle ear during anti-bacterial therapy compared with disease attributable to bacterial infection alone (Chonmaitree et al., 1990). It is unclear whether the decrease in efficacy of antibiotics is due to impaired host responses (poor neutrophil function) or due to poor penetration of antibiotics into the middle ear. In a recent clinical trial heptavalent pneumococcal vaccine had 90% efficacy for prevention of bacteremia and 57% efficacy for prevention of AOM caused by serotypes included in the vaccine. However, the frequency of AOM caused by pneumococci not included in the vaccine increased (replacement phenomenon), so that the overall effect of the vaccine was reduced (Eskola et al., 2001). In contrast to the pneumococcal vaccine, viral vaccines seem very effective in preventing AOM. Belshe and colleagues reported considerable efficacy for an attenuated live influenza vaccine in preventing AOM, and RSV prophylaxis with RSV antibodies was also associated with a marked decrease in otitis media (Belshe et al., 1998; Group, 1998a).

4.3. Determinants of adenovirus tropism

The human adenoviruses consists of over 51 known serotypes which have been divided into six subgroups (A–F) with distinctly different organ tropism. Although other factors may influence infectivity and replication of these viruses, high affinity attachment of virions to host cell receptors represents a key determinant of tissue tropism. Examples of this distinctly different organ tropism are a predominance of subgroup A adenoviruses (Ad) such as Ad31 in pneumonia in patients with primary immunodeficiencies; the preference of subgroup B viruses such as Ad11, 34 and 35 for the urinary tract in patients with kidney transplants; and the predominance of subgroup C viruses in hepatitis in liver transplant patients. Receptor binding is mediated by the Ad fiber protein, a homotrimeric molecule composed of an amino terminal region that anchors the fiber to the penton base capsid protein, an elongated central shaft domain (van Raaij et al., 1999), and the carboxy-terminal receptor binding knob. A high resolution structure of the Ad12 fiber knob bound to its receptor, the coxsackie-adenovirus receptor (CAR), has recently been obtained by x-ray diffraction (Bewley et al., 1999), and amino acid residues directly involved in receptor binding were defined for several adenoviruses through mutagenesis studies (Roelvink et al., 1999). Adenoviruses differ remarkably in the isoelectric point of the fiber protein knob domain, e.g. pH 8.9 for Ad8 versus pH 4.5 for Ad35, suggesting that these fiber proteins cannot use the same receptor. Besides CAR, heparin sulfate proteoglycans (Dechecchi et al., 2000) and sialic acid (Arnberg et al., 2000) mediate adenovirus attachment. Since CAR is only expressed on the basolateral but not the luminal surface of epithelium, CAR can probably not be used to target adenoviral vectors in the therapy of cystic fibrosis. The important role that charge plays in virus-cell interactions can be deduced from the differential effect that removal of sialic acid from the cell surface by neuraminidase treatment has on adenovirus attachment. While Ad19p attachment is not affected by neuraminidase treatment, Ad5 attachment is increased and Ad37 attachment is decreased. The

difference in pH optima may well be a determinant of tissue tropism. The major pathogens in adenoviral eye infections, Ad8, Ad19 and Ad37, belonging to subgroup D, are very similar in their knob charge. Chimerization of fiber proteins, whether evolved in nature or generated by mutagenesis, can cause a significant change in adenovirus pathogenesis. In 1984, a new Ad7 genotype (Ad7h) appeared in Argentina, Uruguay and Chile, where it caused severe respiratory tract infections in children. Analysis of the Ad7h fiber protein revealed that it was a chimera containing Ad7 and Ad3 sequences. In 1996 this new genotype and Ad7d2 also emerged in Japan, where Ad7 had been absent for 30 years, and caused outbreaks of respiratory disease.

The fiber protein, however, is not the only factor that determines adenovirus tropism. Virus uptake is thought to also be mediated by an interaction between the penton base protein with integrin αvß 3 or αvß 5. Adenoviruses also differ in their ability to induce inflammatory responses. While Ad 7 is a potent inducer of interleukin 8 (IL-8), a hallmark cytokine of viral pneumonia, Ad5 is not; this might explain why Ad7 but not Ad5 causes significant respiratory disease. In summary, it can be concluded that neither subgroup classification alone nor fiber protein knob charge alone determine adenovirus tissue tropism. Key amino acids in the knob, as well as certain motifs of the penton base protein and possibly other adenovirus proteins interact in virus attachment and internalization.

4.4. Pathogenesis of influenza A (H5N1) virus infection in a primate model

Avian influenza viruses of the H5N1 subtype were found to be transmitted directly from poultry to humans in 1997 in Hong Kong. These viruses were highly pathogenic in chickens and also caused severe clinical symptoms in humans, leading to the death of six of 18 infected individuals. In order to obtain a better understanding of the pathogenesis, tropism and kinetics of replication of these viruses in primates, cynomolgus monkeys (*Macaca fascicularis*) were infected with the highly pathogenic H5N1 A/Hong Kong/156/97 isolate that was obtained from the index case. Four monkeys were inoculated with 2.5×10^4 $TCID_{50}$ in a 5 ml inoculum that was administered intratracheally, orally (tonsils) and onto the conjunctiva. Two of the monkeys were euthanized on day 4 and the remaining two on day 7 post infection. The two monkeys that were euthanized on day 7, post infection developed a respiratory distress syndrome with high respiratory rate and fever on day 5. By day 7, one of the monkeys was lethargic and severely ill, with central cyanosis. The other monkey was also ill and developed fever. The virus replicated to a titer greater than 10^6 $TCID_{50}$ per g lung tissue on day 4 but could not be isolated on day 7. Macroscopic lung pathology was dominated by peribronchial consolidation and necrotic lesions. Histopathologic examination of the tissues collected 4 and 7 days post infection revealed extensive pathologic changes in the respiratory tract characteristic of a viral necrotizing interstitial pneumonia. Although RT-PCR for H5N1 influenza virus was positive not only in the respiratory tract but in spleen, heart and also the cerebrum and cerebellum of one monkey, virus could only be demonstrated by immunoperoxidase staining in, and isolation from, the respiratory tract. The respiratory tract seems to be the major and probably the only target for the H5N1 influenza virus. Although cynomologous monkeys have been used earlier as a model for H3N2 influenza disease (Rimmelzwaan et al., 1997), this is the first time a primate model for H5N1 viruses has been described. Influenza H5N1 clinical symptoms observed in this study correlate well with what was seen in human disease caused by these highly virulent viruses, and was more severe than what is seen with H3N2 viruses. Therefore, cynomologous monkeys may provide a useful model for studying influenza H5N1 pathogenesis and for developing H5N1 vaccines.

4.5. SB-242235, an orally active, selective p38 mitogen activated protein (MAP) kinase inhibitor improves pulmonary functions in a murine influenza pneumonia model

Influenza infections are responsible for significant morbidity, especially in high-risk groups with

underlying cardiopulmonary disease and in the elderly. The pathology results from a vigorous inflammatory response in the respiratory tract and damage to respiratory epithelial cells. In cells exposed to inflammatory cytokines, p38 mitogen activated kinase (MAP) activation leads to the upregulation of cytokines such as IL-6, IL-8 and TNFα (Ono and Han, 2000). A recent study examined the effect of SB-242235, a highly selective orally bioavailable inhibitor of p38 MAP kinase, on pulmonary function in mice infected with influenza A virus. Mice were infected intranasally with influenza A/PR8/34 and treated with SB-242235 at different time points post infection. Initiation of treatment on day 0, 3 or 5 post-infection resulted in a 57% ($P < 0.01$), 32% ($P < 0.01$) or 10% improvement in pulmonary capacity, respectively, compared with placebo-treated control animals. No effect on virus clearance, survival, or antiviral immunity was observed. The efficacy of SB-242235 in reducing pulmonary resistance and increasing blood oxygenation was similar to that of the neuraminidase inhibitor oseltamivir, the steroid dexamethasone, and the COX-2 inhibitor nimesulide. SB-242235 was superior to non-specific NSAIDs indomethacin, naproxen, and ibuprofen. In mice and ferrets, SB-242235 reduced airway neutrophilia, and treatment was well-tolerated without any adverse effects. These data suggest that p38 MAP kinase is involved in influenza-induced cell signaling and that inhibition of this enzyme might reduce the severity of pulmonary disease.

5. Vaccinology and prevention

5.1. What are the respiratory virus diseases requiring vaccines and what vaccines are available?

Fortunately, the number of viruses that cause respiratory disease severe enough to require hospitalization is limited. In children younger than five years, RSV (subgroup A and B viruses), the parainfluenza viruses (PIV1, -2 and -3), influenza A and B, and adenovirus types 1, 2, 3 and 5 are the major respiratory pathogens. Influenza A and B remain, due to antigenic drift and shift, important agents in all age groups with very severe disease most commonly occurring in the elderly. RSV is the single most important cause of severe respiratory disease in infancy and childhood but it also causes significant morbidity in the elderly and in immunocompromised individuals. Kim, Chanock, Brandt and Parrott were the first to quantify the contribution of these viruses to the severe respiratory tract disease leading to hospitalization of infants and young children. RSV, PIV3, PIV1, PIV2, adenoviruses and influenza B caused 23, 12, 6, 3, 6, and 1%, respectively, of respiratory disease leading to hospitalization, while influenza A was responsible for 3% between 1957 and 1968 (H2N2 era) and 7% between 1968 and 1976 (H3N2 era) (Brandt et al., 1972; Parrott et al., 1973; Kim et al., 1979; Murphy et al., 1988).

What are the general principles underlying vaccine development for these viruses? First, the protective antigens of the virus and mediators of immunity to reinfection are largely known. It is generally accepted that neutralizing antibodies to surface glycoproteins (G and F of RSV, HN and F of PIV, and HA and NA of influenza) or to capsid proteins (hexon and fiber proteins) of adenoviruses are the major mediators of resistance to reinfection. Second, serum and mucosal antibodies make independent contributions to immunity against reinfection. Whereas serum antibody is needed to mediate immunity in the lower respiratory tract, mucosal antibody is needed to protect the upper respiratory tract (with the exception of adenovirus, where serum antibody alone can prevent URI). Third, live virus vaccines are generally more immunogenic than nonliving virus vaccines in immunologically naïve subjects, but identifying a live vaccine virus that is both safe and sufficiently immunogenic can prove to be a difficult task. Fourth, different age groups might need different vaccines. Infants younger than four months of age make less antibody than older children due to immunosuppression by maternal antibodies and immaturity of the immune system, and only potent immunogens, e.g. live virus vaccines,

will be successful in this population (Murphy et al., 1986). On the other hand, a live virus vaccine that is sufficiently attenuated for use in infants might be over-attenuated in the elderly (Gonzalez et al., 2000). Fifth, viruses differ in their ability to cause disease in the presence of maternal serum antibody. While mucosally delivered RSV, PIV3 and influenza are infectious in the presence of maternal antibody, PIV1, PIV2 and adenovirus seem to be restricted in their replication in young infants by maternal antibodies. These five principles need to be considered when determining the optimal vaccination strategy (systemic versus mucosal, vectored antigen versus live attenuated virus) and the target age for vaccination.

Which vaccines are available? Against influenza, the vaccine currently in use in the US is mostly a non-living, subvirion vaccine made from egg-grown purified virus disrupted with detergents. For practical purposes, the only relevant protein in the vaccine is the influenza hemagglutinin. The strains to be included in the vaccine for annual vaccination are selected by the Public Health Service based on the antigenic profile of the currently circulating strains. Subivirion or subunit vaccines plus adjuvant are also being developed (Minutello et al., 1999; Gluck et al., 2000). Live attenuated virus vaccines based on cold-adaptation of influenza A strain A/AA/6/60-H2N2 and influenza B strain B/AA/1/66 have been evaluated in Phase III clinical trials and should soon become available as a mucosal vaccine administered by nasal spray (Maassab and Bryant, 1999).

To date, an RSV vaccine has not been licensed. Two non-living virus vaccines against RSV are being developed. One is a purified F protein subunit vaccine and the other consists of a G protein-specific peptide conjugated to the albumen-
binding site of the streptococcus G protein (Cano et al., 2000). The purified F subunit vaccine is being evaluated for use in seropositive populations such as the elderly or high-risk older children (Gonzalez et al., 2000). Used by itself, the F subunit vaccine has the disadvantage of not inducing a potent mucosal antibody response. Also, this antigen induces a serum antibody response that is dominated by non-neutralizing antibodies (high ratio of titer of F protein-specific binding antibody to titer of neutralizing antibody).

Subgroup A and B live RSV vaccine candidates are being developed (Collins et al., 1999). One candidate vaccine bearing multiple attenuating mutations has been evaluated in 1–2 months old infants and was found to be attenuated and immunogenic but retained mild reactogenicity for the upper respiratory tract (Wright et al., 2000). It is likely that recombinant cDNA technology will be used to develop or improve live attenuated RSV vaccines in the near future (Whitehead et al., 1999a). For example, a subgroup B RSV vaccine candidate was developed by substituting the G and F glycoprotein genes of RSV B for the corresponding genes in an attenuated recombinant subgroup A virus (Whitehead et al., 1999b), thereby rapidly generating a live attenuated subgroup B RSV vaccine candidate.

To date, a PIV vaccine has not been licensed. Subunit and vectored vaccine candidates against PIV3 have been developed and tested in animal models, but there are no reports of ongoing clinical trials. Two live-attenuated PIV3 vaccine candidates, a bovine PIV3 (BPIV3) and a cold-adapted PIV3 (*cp*45), have been evaluated in clinical trials, and both viruses were found to be safe and immunogenic in seronegative infants (Karron et al., 1995, 1996). Recombinant versions of these two viruses are also being evaluated as vaccines against PIV3 and as vectors for the expression of foreign viral glycoproteins such as RSV G or measles HA (Karron et al., 1995; Durbin et al., 2000; Schmidt et al., 2000). The recombinant PIV3*cp*45 vaccine candidate is currently being tested in phase II trials in seronegative infants.

For adenoviruses, a live virus vaccine against serotypes 4 and 7 has been used successfully in military recruits from 1971 to 1998. The vaccine made use of site-specific attenuation, i.e. a wt virus capable of inducing respiratory disease if administered to the respiratory tract was administered orally in an enteric coated capsule. The vaccine virus replicated only in the gastrointestinal tract and did not spread to the respiratory tract. The oral vaccine was found to be com-

pletely attenuated and highly efficacious in inducing serum antibodies and protecting the upper and lower respiratory tract (Couch et al., 1963; Howell et al., 1998). A similar tetravalent vaccine against serotypes 1, 2, 3 and 5 should be evaluated for use in young pediatric patients since these four viruses are responsible for over 80% of the adenovirus-related respiratory tract disease in this population.

5.2. Impact of reverse genetics and other systems for manipulation of viruses to be used in vaccines

In virology, reverse genetics refers to the ability to rescue infectious virus from cDNA. Reverse genetic systems have been developed for all major viral respiratory pathogens, i.e. influenza A virus, paramyxoviruses, adenoviruses and most recently coronaviruses. This short review will only address the impact of reverse genetics on vaccine development for selected members of paramyxoviridae. For RSV, the generation of attenuated vaccine viruses started with conventional biological methods. First, wild-type RSV was passaged extensively at low temperature, and the resulting cold-passaged (*cp*)RSV was demonstrated to be attenuated in chimpanzees, as well as in seropositive adults and young children (Friedewald et al., 1968). The *cp*RSV virus was further modified by two rounds of chemical mutagenesis and biological selection for temperature-sensitivity (Crowe et al., 1994a). A panel of resulting mutant viruses was evaluated in mice and chimpanzees and ranked according to increasing attenuation. Several promising candidate vaccines then entered clinical trials. RSV *cpts*248/404, a cold-passaged virus with two additional attenuating point mutations, is the most promising vaccine candidate tested so far, but it still causes transient nasal stuffiness in infants (Wright et al., 2000).

The development of a reverse genetics system for RSV provided a method for expediting development of further attenuated viruses. As a first step, the biologically derived mutant viruses were sequenced completely and their mutations were identified and evaluated by introduction, individually or in combination, into wt recombinant (r)RSV. The direct identification of attenuating mutations made it possible, for example, to improve the above mentioned *cpts*248/404 mutation by incorporating one or more additional attenuating mutations from other vaccine candidates. Furthermore, in many cases, the amino acid substitutions can be engineered to involve two nucleotide substitutions relative to wt, which should confer increased genetic stability. Similarly, a reverse genetics system for PIV3 was established and used to analyze all 15 mutations in the biologically derived cold passaged PIV3*cp*45 (Skiadopoulos et al., 1999). However, the capabilities of reverse genetics go far beyond the analysis of existing biological mutants. A whole new set of methods for generating attenuated paramyxoviruses has been developed as described above. Gene knock-out mutations, i.e. the deletion of non-essential genes, such as the RSV SH, NS1, NS2, M2-2 or G gene, generated a number of vaccine candidates with different levels of attenuation. As another example, in some cases an attenuating point mutation was found to involve a residue conserved, for example, between the L proteins of RSV and PIV3 or between the C proteins of Sendai virus and PIV3. Transfer of the attenuating mutation between the heterologous paramyxoviruses resulted in transfer of the attenuation phenotype. Host range restriction could be employed as a basis of attenuation by creating antigenic chimeric viruses that use an animal virus as a platform for the expression of human RSV or PIV glycoprotein protective antigen genes (Buchholz et al., 2000; Schmidt et al., 2000). In this manner, an HPIV3 vaccine candidate was generated by replacing the BPIV3 F and HN glycoprotein genes in rBPIV3 with their HPIV3 counterparts (Schmidt et al., 2000). Thus, the menu of available attenuating mutations for RSV and HPIV3 include numerous attenuating point mutations (which in some cases specify temperature sensitivity and in other cases do not), gene deletions, and host range restriction elements. The attenuating mutations or elements from each menu can be combined as desired to develop appropriately attenuated vaccine candidates.

Reverse genetics also expedites vaccine development because attenuated platforms can be used to make vaccines against additional viruses. For ex-

ample, attenuated derivatives of RSV A2 (subgroup A) can be used to generate RSV subgroup B vaccine candidates by replacing the A2 F and G glycoprotein genes with their counterparts from the B1 strain of subgroup B (Whitehead et al., 1999b). As another example, a live-attenuated vaccine candidate was developed for HPIV1 by replacing the F and HN coding regions of HPIV3 with their HPIV1 counterparts. The resulting virus thus bears the antigenic determinants of HPIV1 in a wt HPIV3 backbone, which can then be attenuated by the introduction of mutations from the HPIV3 attenuation menu.

Reverse genetics can potentially help us to make vaccines that are satisfactorily attenuated but more immunogenic than wt virus. Protective antigen genes can be moved closer to the genomic promoter and codon usage can be optimized for translation in mammalian hosts. Cytokines and/or chemokines can be co-expressed from additional genes (Bukreyev et al., 1999), and reactogenicity can potentially be reduced, for example by ablating the secreted form of RSV G, which might be a decoy for antibody and might also perturb the T helper lymphocyte response. Vaccine specificity can be broadened by adding additional genes to existing vaccine viruses, e.g. the measles hemagglutinin (HA) gene can be expressed from an additional ORF in rHPIV3, generating a vaccine candidate that protects against both HPIV3 and measles (Durbin et al., 2000). This use of recombinant PIV3 as vaccine and as a vector has several advantages over existing vector systems. It reduces the number of viruses to be administrated to infants and enables intranasal administration and thereby mitigates neutralization and immune suppression by maternal antibodies. The eradication of measles could be facilitated by a vaccine that does not include an infectious measles virus, which might cause prolonged infection in immunocompromised hosts. In summary, vaccines can be optimized through reverse genetics in a number of ways, such as the creation of novel combinations of mutations, the fine-tuning of the attenuation phenotype, the provision of a common attenuated platforms, and the generation of multivalent vaccines.

5.3. Predictive value of animal models in RSV vaccine development

RSV was initially isolated from chimpanzees that developed a common cold-like disease in 1956. Since then chimpanzees and other animal models for RSV disease have been studied extensively. Principally, these higher primates have been used for three different purposes: the development of live virus vaccines, the recovery of RSV antibodies, and lastly to evaluate formalin-inactivated and subunit vaccines, and to study enhanced disease following vaccination with formalin-inactivated vaccine. A number of animal models are used to study RSV disease. Among the apes, chimpanzees have been used most extensively for several reasons. They are permissive for RSV, their core body temperature is similar to that of humans, and symptomatic scoring of URI is possible (rhinorrhea score). The permissiveness of chimpanzees makes it possible to perform quantitative virologic studies in the upper and lower respiratory tract over the time course of an acute RSV infection. Also, the restriction of RSV replication in the respiratory tract of chimpanzees correlates well with attenuation in seronegative human infants. In addition, human immunologic reagents can be used in chimpanzee studies. However, only young, carefully raised chimpanzees are RSV seronegative. Other primates used include Old World monkeys such as African greens, rhesus, cynomologous or bonnet monkeys and New World monkeys such as marmosets, tamarins and owl monkeys. Although all these monkeys are semi-permissive for RSV, their core body temperature is higher than that of humans so that the level of attenuation of temperature-sensitive RSV mutants might be overestimated. Although primate studies provide essential data for vaccine development, their use can be difficult. Transport and care of primates, as well as sample collection are difficult and potentially dangerous, and they require well-trained personnel. Primate research centers must provide an environment similar to that of a child-care facility and, therefore, maintenance costs are very high.

Lambs and calves are also used to study RSV infection, mostly because ovine and bovine RSV

causes significant disease in their natural host. Also, these viruses are important economically. Cotton rats, mice and other rodents are used as small animal models. Mice have the advantage of having a body temperature similar to that of humans and immunological reagents are readily available. Also, gene knockout mice, transgenic mice and various inbred strains are available to study pathogenesis. The mouse model is not without difficulties, however. Mice acquire a large portion of their maternal antibodies by suckling, and, therefore, they are not an optimal model for study of immunosuppression by maternal antibodies. Peak RSV titers vary 100-fold among different mouse strains, and subgroup B RSV is poorly infectious and immunogenic in mice. Immune mechanisms may also vary among different mice strains. Rodent models can be used to study quantitative virology, immunology and airway pathophysiology (a model of wheezing), and weight loss can be used as a surrogate marker for RSV disease. Whichever animal model one chooses to study respiratory syncytial virus, it is essential to clearly define whether the ultimate goal of the study is to prepare for clinical vaccine trials or to understand basic mechanisms of disease.

5.4. RSV vaccine candidates in children — an update

Several cold-passaged (*cp*) live attenuated RSV candidate vaccines have been evaluated in clinical trials. Most of these candidate vaccines were derived by further attenuating the original *cp*RSV through chemical mutagenesis and selection of *ts* mutants. The first two vaccines evaluated in the *cpts* lineage — *cpts*-248/955 and *cpts*-530/1009, were either insufficiently attenuated or were transmitted amongst seronegative children (Karron et al., 1996). A study of a more attenuated vaccine candidate, *cpts*-248/404, has recently been completed (Wright et al., 2000). This virus initially was evaluated in chimpanzees where it replicated to a peak titer of only $10^{1.3}$ pfu/ml in the upper respiratory tract. Due to this restriction in the respiratory tract, it was subsequently evaluated in clinical studies. *cpts*-248/404 did not replicate in adults or older children. In seronegative children 6–24 months of age, however, *cpts*-248/404 replicated to a peak titer of 10^4 pfu/ml in the upper respiratory tract and induced an antibody response against RSV F and G glycoproteins. The virus was well tolerated in this age group, and the frequency of upper or lower respiratory tract disease, otitis media or fever was not different from that of the placebo group. Based on these data *cpts*-248/404 was selected to be the first RSV vaccine to be administered to 1–2-month-old infants, which represent the primary target group for vaccination. In this youngest age group, 17 of 24 vaccine recipients developed a clinical syndrome characterized by nasal congestion that occurred most typically between days 8 and 12 and lasted for approximately 24 h. Since young infants are obligate nose breathers, this interfered with feeding and caused fussiness and difficulty in falling asleep. Most vaccine recipients shed approximately 10^3 pfu of RSV per ml nasal wash. Age of the infant or level of maternal antibodies against RSV did nor affect virus shedding, indicating that virus replication in the nasopharynx was independent of these variables. In these young infants, neutralizing antibody responses and IgG ELISA responses to RSV F or G were rarely detected, most likely because these responses were masked by the presence of maternally derived antibody. These young infants did, however, develop serum and mucosal IgA responses preferentially to the RSV G glycoprotein and detection of serum IgA correlated with protection from re-infection with a second dose of vaccine.

Although *cpts*-248/404 is not an acceptable vaccine in one to two month-old infants, several lessons were learned from the study of this vaccine candidate. First, seronegative infants are a much more susceptible host to RSV than chimpanzees. This means that for further attenuated vaccine candidates, the chimpanzee model of RSV infection will not be very useful. Second, only viruses that do not replicate in adults and older children are attenuated enough for vaccination of infants. And third, reverse genetics is needed to further attenuate the existing biological RSV vaccine candidates. Two of these recombinant RSV

vaccine candidates are currently being evaluated in clinical trials. *cpts*-248/404ΔSH was generated by deleting the SH gene from the recombinant *cpts*-248/404 virus, and *cpts*-248/404/1030ΔSH was engineered to contain an additional (1030) mutation in the polymerase protein. Preliminary data suggest that both recombinant viruses are well tolerated in 6–24-month-old children and are not associated with lower respiratory tract illness. Whereas *cpts*-248/404ΔSH replicates as well as its parent biological virus without the SH gene deletion, the *cpts*-248/404/1030ΔSH mutant appears to be much more restricted in its replication in the nasopharynx. The more restricted *cpts*-248/404ΔSH induced both neutralizing and ELISA IgG antibodies in this age group. However, since this virus replicates, as well as *cpts*-248/404, it is likely not to be suitable for vaccination of one to two month old infants. *cpts*-248/404/1030?SH is currently being evaluated in one to two month old infants. In summary, although *cpts*-248/404 is close to an ideal vaccine candidate, further modifications of the virus through reverse genetics, such as addition of the 1030 mutation, are likely to be needed to generate an RSV vaccine that is immunogenic yet attenuated enough to be given to young infants.

5.5. Passive immunoprophylaxis with antibody

Antibody preparations that neutralize free virus have been used as passive immunoprophylaxis to prevent a number of viral diseases, including hepatitis A and B, varicella and respiratory syncytial virus (RSV) disease. The efficacy of antibody preparations in preventing RSV disease was established first in cotton rat and chimpanzee models, and later in extended clinical trials. Therapy of RSV infections with RSV antibodies, however, have so far failed to prove efficacious (Malley et al., 1998; van Woensel and Kimpen, 2000). The most important mechanism of action of antibody preparations is probably neutralization of free virus. This can occur via aggregation of free virus by bivalent or multivalent antibody, via receptor blockade, via antibody-complement lysis, or via fusion inhibition. Receptor blockade is thought to result from steric inhibition of receptor binding rather than binding of antibody directly to the receptor-binding site itself. Even if receptor binding does occur, virus infectivity can be neutralized through fusion inhibition. Most RSV neutralizing antibodies are thought to use this mechanism of action. Although complete antibody is most effective in neutralizing free virus, antibody binding fragments (Fabs) alone can also neutralize. The minimal unit necessary to ablate infectivity is probably a peptide corresponding to one loop of the complementarity-determining region 3 (CDR3). Although neutralizing and non-neutralizing antibodies against infectious virus are often distinguished, it is not clear whether there really are antibodies that bind glycoproteins in virions without neutralizing the virion (Sakurai et al., 1999). Many so-called non-neutralizing antibodies bind purified (conformationally relaxed) RSV F glycoprotein but not the (conformationally correct) F protein on infected cells. Different immunoglobulin subclasses neutralize virus with varying efficacy, and mouse and human IgG subclasses also bind complement with varying efficacy. The replication of Sendai virus (murine PIV1) and influenza A virus can be inhibited intracellularly by IgA and similar effects of IgA on RSV replication may occur. A last but essential determinant of neutralizing activity is defined by the amount of antibody available to neutralize virus. To prevent RSV disease in the lower respiratory tract, a serum neutralizing antibody titer of approximately 1:300 is required (Groothuis et al., 1993; Top et al., 2000). Upper respiratory tract infections, however, can only be prevented with serum antibody titers as high as 1:5000–1:15 000. Such titers can only be achieved in experimental settings in small rodent models. There is no single monoclonal antibody directed against RSV G protein that neutralizes completely on its own but cooperative neutralization occurs when multiple mAbs are used.

Effective treatment for RSV illness is very limited. Corticosteroids, bronchodilators, ribavirin, and, more recently, RSV mAbs have been used but none of these therapeutic interventions are accepted as reproducibly efficacious. It seems that neither antivirals nor anti-inflammatory agents alone improve the outcome of RSV disease. A

single dose of topically administered human Fabs has been shown to clear free virus from the respiratory tract of rodents, but their effect is short-lived since infected cells release newly synthesized infectious virus within a day or two (Crowe et al. 1994b). Whereas peak virus titers in humans usually occur around day 4, most patients with clinical RSV disease likely present no earlier than day 7. At this point, virus load is already in decline and it may be too late for antivirals, and anti-inflammatory drugs such as MAP kinase inhibitors may be more effective in reducing cell injury.

Are there immunological consequences of passive immunoprophylaxis? In chimpanzees therapy with RSV antibodies suppresses the primary antibody response to RSV. This effect is mostly antigen-specific but may also have a non-specific aspect mediated by the Fc portion of the antibody. The secondary antibody response to RSV in chimpanzees is enhanced by prior antibody therapy. However, this effect was not observed in mice or in clinical studies. T cell responses do not seem to be as affected by antibody therapy as humoral responses, and T cells might fill in for absent primary antibody responses. Whether this is a desirable effect or not, remains uncertain.

5.6. Monoclonal antibody for prevention of RSV disease

Without a safe and effective RSV vaccine available, monthly infusion of RSV-IGIV (RespiGam®) was the first effective measure to prevent RSV-induced lower respiratory tract infection (LRI) and hospitalization in infants. The evaluation of RSV-IVIG in cotton rats correctly predicted the serum concentrations necessary to protect against RSV-induced LRI (Prince et al., 1985a). Protective concentrations in children were achieved by monthly infusions of 750 mg/kg of RSV-IVIG. In order to increase the potency of a RSV antibody preparation, and in order to be able to replace intravenous with intramuscular administration, a humanized monoclonal anti-RSV fusion protein antibody was developed. Mab1129, a mouse monoclonal antibody developed in the Laboratory of Infectious Diseases at NIH against the RSV F protein antigenic site A, one of two antigenic sites that are conserved amongst different RSV strains, was selected for humanization based on in vitro and in vivo studies, and the complementarity determining regions (CDR) were transferred from the mouse antibody to a human IgG. This humanized IgG should have pharmacokinetics similar to human IgG and permit repeated administration at monthly intervals. The chosen Mab, MEDI-493, had no cross-reactivity with adult or neonatal tissue, broadly neutralized RSV subgroup A and subgroup B isolates at concentrations of 20 ng/ml, and proved to be 50–100 times more active on a weight basis than RSV-IVIG in the cotton rat model (Johnson et al., 1997). Adult volunteers tolerated doses of the humanized antibody MEDI-493 from 1 to 30 mg/kg well, and only some volunteers developed a low-titer, transient anti-idiotypic antibody response. MEDI-493 had a half-life of 17 days, as is expected for IgG, and serum concentration after IV and IM administration were comparable except for the initial bioavailability. MEDI-493 was safe and well tolerated in phase I/II studies in high-risk children, and had no specific immunogenicity in and of itself. Monthly dosage of 15 mg/kg maintained serum concentrations greater than 40 μg/ml, and a single intravenous dose of 15 mg/kg reduced RSV titers in tracheal secretions of intubated children with RSV infection (Malley et al., 1998).

The IMpact-RSV study that led to FDA approval of MEDI-493, also called palivizumab, was a 2:1 randomized, double-blinded, placebo-controlled phase III multicenter study conducted at 139 sites in the US, Canada and the UK. Study subjects received five doses of MEDI-493 or placebo at intervals of 30 days and were followed for a total of 150 days. The primary endpoint of this study, overall RSV-related hospitalization, was significantly reduced ($P < 0.001$) from 10.6% for placebo recipients ($N = 500$) to 4.9% for MEDI-493 recipients ($N = 1002$). For premature infants with chronic lung disease, the incidence of RSV hospitalization was reduced from 12.8 to 7.9% ($P = 0.038$), and for premature infants without chronic lung disease, it was reduced from 8.1 to 1.8% ($P < 0.001$). Total RSV hospitalization

days, total days with increased oxygen requirement, total days with severe LRI, rate of ICU admission and total days in ICU were secondary endpoints that occurred less frequently in the MEDI-493 treated group ($P < 0.05$). Thus, administration of 15 mg/kg MEDI-493 by IM injection was found to be safe, well-tolerated, and to lead to a 55% reduction of RSV hospitalization in high-risk children (Group, 1998b). This conclusion was confirmed in an outcome survey conducted in nine centers in 1998 and 1999, in which 1839 high-risk children received 15 mg/kg palivizumab. Although direct comparison to the IMpact-RSV study is not possible, RSV hospitalization rates were low and similar to those observed in the IMpact-RSV study: 2.1% (vs. 1.8% in the Impact-RSV study) of premature infants without CLD and 4.0% (vs. 7.98%) of children with CLD were hospitalized in the 1998–1999 RSV season (Sorrentino and Powers, 2000).

5.7. Safety of RSV purified fusion protein-2 vaccine in pregnant women

An alternative strategy to protect neonates and young infants against severe RSV disease is vaccination of pregnant women. Transfer of high concentrations of maternal RSV-specific IgG to the fetus is expected to protect the infant against severe disease (Glezen et al., 1981). The safety and immunogenicity of respiratory syncytial virus purified fusion protein-2 (PFP-2 Wyeth Lederle Vaccine and Pediatrics, Pearl River, NY) were recently evaluated for use in pregnancy. Thirty-five pregnant women were randomized at a ratio 2:1 to receive RSV PFP-2 or saline placebo at 30–34 weeks of gestation and were followed until the time of delivery. Infants were followed during their first year of life and their first RSV season. RSV PFP-2 was safe and well tolerated by pregnant women, and there were no systemic reactions or serious adverse events associated with vaccine administration. All 35 infants were born healthy, and there were no differences in the frequencies and outcomes of neonatal events between the groups. During the first RSV season, there was no increase in the frequency or severity of respiratory tract illnesses in infants of vaccine recipients.

6. Pandemic preparedness

6.1. D.A. Henderson: lessons from the past

There is ample material from which to draw lessons relevant to needed preparations for pandemic influenza. But, to date, the response, and specifically preparations for dealing with a serious pandemic of influenza remain more in the realm of academic reflection than meaningful action. Although much time has been devoted to the development of a national response plan, we do not seem to be much better equipped to deal with a new pandemic of influenza than we were in the spring of 1957, when the H2N2 strain of influenza A virus emerged. At that time, we faced a burgeoning epidemic, whose virulence and propensity for spread were as yet unknown. A program of surveillance and field epidemiology to better define the epidemic had to be developed and, at the same time, preparations were needed for distribution and use of a new influenza vaccine, if it arrived in time. It did not. It appears today that not much progress has been made over the past 30 years, and still no real sense of urgency in dealing with the essentials of the problem can be felt.

Although much has been written about the 1918 epidemic, it is still perceived by most as an interesting but questionably relevant tale of death and disease during an earlier, pre-antibiotic era of medicine. Many doubt that an epidemic of this severity would be possible today. But, here are a few of the facts. First, it is important to recognize that, for the US, it dwarfed all other outbreaks of the 20th Century. At that time, more than 20 million died worldwide and in the United States, there were more than 500 000 registered deaths. From various studies, it is thought that, overall, perhaps 40% of the population became ill, of whom about 2% died. Medical services were overwhelmed but, other than supportive care, there was little that curative medicine could offer. The deaths were so numerous that burials were greatly delayed because of the lack of morticians and grave diggers. Pictures from the time provide at least a pale illumination of that catastrophic period.

The effect of the disease was anything but uniform. Surveys revealed morbidity rates ranging from 15% to more than 60% in different parts of the country. Some remote and rural areas escaped the disease entirely but in some areas, it was remarkably lethal. Western Samoa, then a New Zealand protectorate, registered 8500 deaths in a population of 38 000. This represented 22% of the entire population. Curiously, American Samoa, only 50 miles away but with a quarantine in effect, was one of the few political entities to escape the epidemic entirely.

What was surprising and unique about the 1918 epidemic was that more than half of all deaths were in persons between 15 and 45 years of age — an age bracket in which death is a relatively uncommon phenomenon. Pregnant women and those with cardiac problems were at highest risk but most, who died, were otherwise in good health. A substantial number in this age group died of a fulminant, rapidly progressive pneumonia marked by severe cyanosis with death occurring within a matter of 1–3 days, in brief, what seemed to be almost certainly, a primary influenza pneumonia that would have benefited little from antibiotics, had they been available.

Today, a better outcome might be foreseen with better ventilatory support in intensive care units; with the administration of antiviral agents; and with the administration of antibiotics for the treatment of secondary pulmonary infections. But under epidemic circumstances, the bulk of cases in a new pandemic would occur over a period of only 3–6 weeks and only a fraction of patients could be accommodated in suitable hospital settings, let alone appropriate intensive care units. What quantity of antiviral drugs is available for emergency use? Where do we obtain the added quantities of the standard antibiotics that today, are produced on a just-in-time basis — when, during a period when clinical facilities are not stressed, we are regularly experiencing antibiotic shortages on a regular, rotating basis.

A cogent question is whether under epidemic circumstances, our modern health care system could provide a standard of clinical care that would be better than it was in 1918. But there is still another dimension to the problem. Do we appreciate that a 'pandemic', so characteristic of the emergence of a new influenza strain, means exactly that — a worldwide epidemic. Many countries — developed and developing, have made no preparations and will inevitably turn to those with resources to help them in dealing with a catastrophe — a catastrophe that poses an international security threat.

The rapid production and administration of large volumes of vaccine effective against the emergent new strain has been a basic building block of the strategy for dealing with pandemic influenza and, understandably so, given the limitations of curative medicine and the capacity of clinical services. The first real test of this strategy came during the 1957 epidemic. The first notice of a major outbreak occurred in mid April; specimens were received in the US on 13 May; and field testing of new lots of the vaccine began in July. Not a bad record. It was foreseen that 60 million doses would be required. With good fortune in adapting the virus to grow at reasonably high titer on egg membrane and provided that sufficient fertilized eggs could be obtained, it was expected that a production target of 1 February could be met. Even this was a problematical date given that the seasonal peak of epidemic influenza usually occurs between the end of December and the end of February. Still, it was believed that substantial numbers would be able to be protected.

However, 1957 was not a typical year, and such, one must note, was the case when the 1918 epidemic strain first appeared. Widespread epidemics began occurring in mid to late September, two months before they were anticipated. More than half of all counties reported epidemics by mid-October and by the end of October, the peak incidence had past, long before any substantial quantity of vaccine was available. Given these experiences, could we expect another new strain to behave differently today?

Over the past 30 years, extensive studies have been conducted in the search for a satisfactory live attenuated vaccine and for various approaches in production which would permit new antigenic variants to be produced rapidly and in quantity in tissue cell culture. However, today we

are still producing influenza vaccine in the allantoic cavity of hens' eggs, as we were in 1957; procuring adequate supplies of fertile eggs in a timely manner remains a serious problem; and difficulties in adapting new strains to eggs remain.

Were we able to solve the vaccine production problem for our own country, this would not be the end to the practical quandary of dealing with the pandemic. Few countries have influenza vaccine production capability and the international implications of the US having all or much of the vaccine supply are profound. Following the 1976 swine influenza outbreak at Fort Dix, New Jersey, the US had embarked on a program to rapidly produce an appropriate vaccine. Vaccine production capability in Europe and other countries was so marginal that a World Health Organization Committee could only recommend that the Fort Dix situation be carefully monitored — a strategy of wait and hope. But as those at the WHO meeting commented in corridor conversations, they would not wish to face the ethical and political dilemma the United States would face were there a pandemic and the US was the only nation with a vaccine.

In recent meetings with national and local hospital authorities, current capabilities of the medical system in US to deal with sudden surges in demand such as might follow release of a biological weapon were explored. Such a release would result in an epidemic that would stress the system not unlike the way it would be stressed by a pandemic of severe influenza.

From these meetings, it was evident that the elasticity of the nation's bed supply has been significantly reduced as drives for financial efficiency and the increasing use of out-patient procedures have sharply reduced the numbers of beds in all hospitals. Meanwhile, managed care-driven market pressures and federal government reimbursement reductions have driven large numbers of hospitals into operating deficits. Many of the municipal hospitals, once a primary source of care for the less prosperous and uninsured have been privatized. Meanwhile, the hospitals are experiencing severe labor shortages, especially for nursing and technical personnel. Few have either the resources or motivation to prepare to respond to the challenges posed by mass casualties due to any cause.

Reserves of antibiotics, as noted earlier, are marginal to nil; the public health infrastructure needed to deal with epidemic disease is grossly understaffed, underpaid and under trained; mechanisms for the development and implementation of community-wide plans are largely unexplored. It is not unreasonable to suggest that we are today less well-prepared to deal with an epidemic of influenza than we were 30 years ago in most parameters that one can identify.

What might a new strain of influenza mean in terms of numbers of cases and deaths. Estimates of past pandemics suggest that perhaps 40% of the population were affected in a first wave. With rising proportions of the population in urban areas, the greater and more rapid mixing of populations through travel, a figure of not less than 60% would seem more reasonable. Thus, in a city of 3.0 million, one might expect 1.8 million cases of influenza. It is doubtful that either antibiotics or antiviral agents would be of much help given the number of cases and the dearth of reserve supplies. The number of deaths would vary greatly depending on the strain. In Hong Kong, a recent new strain, H5N1, resulted in death among six of 18 persons infected. It did not spread readily, however. It is estimated that the 1918 strain killed 2% of those who became ill, while the 1957 case-fatality rate was about one-tenth as large or 0.2%. Thus, in a city of 3 million persons, one might anticipate between 3600 and 36 000 deaths over a period of 3–6 weeks.

Medical care would consist largely of supportive therapy given the numbers of those ill. Public health measures, likewise, would consist of little more than reassurance given the likelihood that vaccine supplies would not be available and that stocks of antiviral drugs would be too small to be of significance. Many would argue for the closing of schools, churches and other places of public gathering but experiences suggests that such actions produce little benefit. The wearing of masks was once a favored intervention but this, too, has been discredited. In brief, without vaccine, there would be little that could be done of practical benefit except to reassure the community that the

epidemic would someday pass and to accept the criticism of the public and political leadership for failure to make reasonable preparations.

At least four lessons can be derived from past experience. First, the threat of pandemic influenza caused by an especially virulent strain is a continuing threat that has to be taken seriously. Second, adequate supplies of an effective vaccine, available in a timely manner, are absolutely critical to a preventive effort. Solving this problem should command top priority for research and development funding. Third, special plans, programs and funding are needed within the health care system to permit development of an adequate community-wide response to the occurrence of mass casualties whatever the cause. Lastly, additional research in influenza is needed to better understand its pathogenesis and epidemiology with the expectation that better preventative measures might eventuate.

6.2. Vaccine development for potential pandemic strains of influenza virus

Pandemics are the most dramatic presentation of influenza A virus and they cause considerable excess mortality as a result of pneumonia and exacerbation of cardiopulmonary or other chronic diseases. The epidemiologic success of influenza A is in large part due to antigenic variation that takes place in the two surface glycoproteins of the virus, i.e. its hemagglutinin (HA) and neuraminidase (NA) proteins. To date, 15 HA and 9 NA subtypes have been defined and are used to classify influenza A viruses. Antigenic variation occurs either gradually through accumulation of point mutations (antigenic drift) or more abruptly though introduction of a new HA gene into virus circulating in the human population, be it through reassortment of animal and human viruses or through a change in host-specificity of an animal virus (antigenic shift). A 'pandemic virus' can be defined as a virus with a new HA with or without a novel NA gene, acquired through antigenic shift, that spreads readily from person-to-person in a population that is highly susceptible to infection. The 20th century saw three pandemics caused by new HA subtypes. Influenza A H1N1 caused the 'Spanish flu' in 1918, subtype H2N2 ('Asian flu') was the causative agent of the 1957 pandemic and the H3N2 subtype ('Hong Kong flu') caused a pandemic in 1968. The excess mortality for the 1918, 1957 and 1968 pandemics in the US alone can be estimated to have been 500 000, 70 000, and 34 000 deaths, respectively. If one applies mathematical modeling to estimate the effects of a future pandemic, the US alone can expect between 89 000 and 207 000 excess deaths in the next pandemic (Meltzer et al., 1999).

What are the viruses with pandemic potential? In 1997, 18 individuals in Hong Kong were infected with H5N1 influenza, an avian subtype earlier not known to infect humans (Subbarao et al., 1998). Of the 18 patients 1–60 years of age, six died. Molecular analysis established that all eight genes of the H5N1 virus were of avian origin and that reassortment with human viruses had not occurred (Subbarao et al., 1998). The reported 18 infections were most likely acquired from poultry; human-to-human spread, however, was a rare event. Sequence analysis of the Hong Kong H5N1 virus suggests that it is a reassortant made up from two or three different avian parent viruses: HA from a goose H5N1 virus (Xu et al., 1999), NA from a teal H6N1 virus (Hoffmann et al., 2000), and internal genes from a quail H9N2 (Guan et al., 1999) or a teal H6N1 virus (Hoffmann et al., 2000). Although this virus did not spread readily from person to person, deep concern was caused by the fact that this was the first known avian influenza A virus that caused disease in humans. Until this outbreak it was thought that the receptor specificity of avian HA proteins limited their infectivity to avian species. This notion, however, needs to be revised. In March 1999, H9N2 influenza A viruses were isolated from two children with febrile upper respiratory tract illness (Peiris et al., 1999). This virus, again, was an avian virus that was able to infect humans without passage through an intermediate host (Lin et al., 2000).

What are our options for vaccination against potentially pandemic viruses? In the case of the H5N1 viruses, there are four options for generating an effective influenza vaccine. A conventional inactivated vaccine can theoretically be generated

by reassortment of internal genes from influenza A/PR/8/34 with H5N1 glycoprotein genes. There are, however, problems with incompatibility between certain gene segments. Secondly, a cold adapted live attenuated vaccine approach could be taken. The influenza A/Ann Arbor/6/60 cold-adapted strain has been used to generate an H5N1 vaccine in which the HA cleavage site was modified (Li et al., 1999a). This virus could also be used to produce an inactivated vaccine. Thirdly, a surrogate virus that is nonpathogenic but antigenically related (Takada et al., 1999) could be sought. One of the closest antigenically related viruses found to date was of the H5N3 subtype, but this virus was not highly immunogenic in humans. Fourthly, purified protein could be used but again limited immunogenicity may be a problem.

The development of vaccines against potential pandemic viruses poses a number of challenges. To begin with, most of the work has to be conducted in biosafety level 3+ laboratories. There is a large array of avian viruses that could potentially become pandemic viruses and those with a genotype that confers transmissibility have to be identified. H5N1, H9N2 and H6N1 viruses that were the genetic precursors of the H5N1 and H9N2 viruses that caused human infections in Hong Kong (Guan et al., 1999; Xu et al., 1999; Hoffmann et al., 2000) and that continue to circulate among birds should certainly be given priority in vaccine development. An adequate strategy for vaccine development has to be selected, and safety testing must be expedited in mice, ferrets, chickens, and eventually humans. Use of recently described plasmid based reverse genetics systems may lessen the technical challenges faced in generating vaccine candidates. Assays to evaluate immunity have to be optimized (hemagglutination inhibition assays do not perform well with avian HA proteins, yet neutralization assays are time consuming and technically more difficult), and serologic and molecular diagnostic reagents must be made available. Collaboration between veterinary and public health authorities must be maintained and new technologies such as plasmid based reverse genetics systems must be used. Last but not least, alternative substrates for vaccine production and new adjuvants are urgently needed.

6.3. Concern for preparedness: maintaining interest

Although influenza epidemics occur every winter, we have only had three pandemics this past century — 1918, 1957 and 1968. While annual influenza epidemics generally cause an excess mortality of 20 000–40 000 people in the US, it is only the pandemics that remain in memory. Of the three pandemics that occurred in the twentieth century, the 1918 pandemic was the most devastating and claimed the most victims. Although there is wide spread public interest in the 1918 pandemic, the event is mostly remembered as an interesting event in the distant past. Whether due to denial or not, few feel that an influenza pandemic is a current threat.

Most journalists see their role not only as educators and advocators but also as entertainers. The interest of the readership has to be captured, and it can only be maintained if their curiosity is satisfied. While the AIDS pandemic and its effect on people's lives found a wide audience through most of the early 1990s, the late 1990s were characterized by fatigue and a reduced interest in the pandemic (although it was still on the increase). As a result, editors were much less willing to include related stories. Reportage on influenza pandemics suffers a similar fate: the disease itself is old, and the last pandemic occurred too long ago for most to remember. The mere warning of a coming pandemic is almost perceived as an unfulfilled promise if the event does not occur shortly after the article is published. Breast cancer, in contrast, is one of a few examples, where interest in a disease process can be maintained over an extended period of time. The risk of suffering from breast cancer is felt much more acutely, it seems, and there is a sense that proactive behavior will lead to an improved outcome. Whether the mass media will take on a role in changing the public's attitude toward influenza as a threat is very much in doubt as long as journalists themselves perceive the next pandemic as an event as anonymous and inevitable as an earthquake.

7. Antivirals and other management strategies

7.1. Rho protein — a new target for RSV antivirals

RSV infection is initiated by G glycoprotein attachment to cell surface receptors and followed by virus-cell fusion that is mediated by the F protein. The cleavage-activated RSV F1 protein is thought to interact with the target cell membrane through its N-terminal fusion peptide, which is released from a shielded position within the F homotrimer through a major conformational change. Insertion of the F1 N-terminus into the cell membrane destabilizes the cell membrane and induces lipid mixing that is followed by mixing of contents, thereby enabling the viral nucleocapsid to enter the cytoplasm. The RSV F protein was recently found to interact with a small GTPase called RhoA through a domain that lies just carboxyterminal to the F1 fusion peptide (Pastey et al., 1999). RhoA is a member of the ras superfamily that is expressed intracellularly in all cell types. It induces bundling of actin filaments into stress fibers, focal adhesion plaque formation, cell-to-cell adhesion and organization of integral membrane proteins; it has additional roles in cell morphology and motility as well as cell cycle transition from G1 to S.

A series of overlapping RhoA peptides from the interaction domain were evaluated in RSV plaque reduction neutralization assays. RhoA peptide 77–95 has an IC_{50} of about 1 μg/ml against an inoculum of 100 pfu. Neutralizing activity is also seen against parainfluenza virus type 3 (PIV3), but not against a variety of other paramyxo, orthmyxo, corona and filoviruses (Pastey et al., 2000). Intranasal administration of 500 μg of peptide 77–95 prior to intranasal infection of mice with RSV reduces RSV replication more than 100-fold and also diminishes weight-loss. Studies with recombinant RSV expressing green fluorescent protein indicated that RhoA peptide 77–95 inhibited RSV replication at a very early stage of infection. Subsequently, it was shown that fusion of cells transfected with RSV F, G and SH was inhibited by this peptide, suggesting that it exerted an effect on RSV replication at the level of membrane fusion or entry. Since RhoA is an intracellular molecule, one would have to hypothesize that the interaction of the N-terminal heptad repeat and fusion peptide with the target membrane causes enough membrane disruption to allow an interaction between RhoA and RSV F. Alternatively, RSV F may not interact with RhoA during the entry process, and the RhoA-derived peptides may simply disrupt the F structure or function to render the virus noninfectious. The question of whether RhoA and RSV F interact during a natural infection is not yet resolved.

RhoA can be found in the cytoplasm bound to GDP and a guanine nucleotide dissociation inhibitor, and, upon GTP exchange and isoprenylation, RhoA associates with the cell membrane. If the proposed model of RhoA-RSV F interaction at the membrane is correct, then inhibition of isoprenylation should inhibit RSV infection. Indeed, inhibition of isoprenylation through HMG CoA reductase inhibitors, such as lovastatin, inhibits RSV infection of HEp-2 cells at an IC_{50} of 3 μM and reduces peak RSV titers in lungs of mice greater than 100-fold with oral gavage of 1 mg per day (Gower, T.L., Graham, B.S., submitted). The antiviral effect of lovastatin is also seen with PIV3 in vitro.

Although it has not been formally proven that RSV F interacts with RhoA in the infected cell membrane, RhoA signaling activity is triggered in RSV-infected cells (Gower, T.L. et al., submitted). While RhoA signaling is not required for RSV replication, syncytium formation is diminished when RhoA signaling activity is inhibited. In addition, RhoA signaling may play a role in other aspects of RSV pathogenesis. RhoA kinase inhibitors reduce the transcription of IL-6 and IL-8 mRNA in RSV infected cells (cytokines abundant in the nasal secretions of RSV-infected patients), and obviate airway hyperresponsiveness, one of the cardinal symptoms of RSV disease (Hashimoto, K. et al., submitted).

7.2. Therapeutic effect of mycophenolic acid on RSV in Balb/c mice

The Balb/c mouse model was used to explore whether mycophenolic acid (MPA), an inhibitor

of de novo purine synthesis in T and B lymphocytes, could improve the outcome of RSV disease, which was monitored by clinical symptoms such as ruffling of fur, increased respiratory rate and weight loss. Oral administration of 100 mg/kg MMF (the prodrug of MPA) daily from day 1 to 6 post-infection reduced weight loss on day 7 post-infection from 23% in untreated animals to 8% in MPA treated mice ($P < 0.001$). A reduction of weight loss (7.8%) was also observed when initiation of treatment was delayed until day 5 post-infection. Virus titers in lungs of MMF treated mice were similar to those of untreated controls but histological changes were reduced. On day 7 post-infection, IFNγ levels were elevated 2.5-fold in the treatment group while IL4, IL5 and IL10 levels were unchanged, indicating a shift toward a T helper cell type 1 response that is thought to correlate with improved RSV disease outcome. These data suggest that inflammatory responses contribute to RSV disease in mice and that an immunomodulatory approach to the treatment of human RS virus disease is worth further consideration.

7.3. Low molecular weight antivirals with picomolar activity against RSV

Using a cell-based assay to identify compounds which are able to inhibit fusion of HeLa cells infected with RSV, more than 300 analogues of a lead compound were synthesized and one compound (termed R170591) was selected for further evaluation. The in vitro 50% inhibitory concentration (IC_{50}) of this benzimidazole derivative (MW 395) was150 pM, and thus its potency exceeds that of ribavirin almost 100 000-fold ($IC_{50} = 10$ μM). R170591 exhibits in vitro antiviral activity against human RSV (subgroup A and B) and bovine RSV but not against pneumovirus of mice or other paramyxoviruses. RSV-induced cytopathic effect was reduced by R170591 at 0.1 nM, and concentration of 10 nM reduced RSV titers 1000-fold in multi-cycle growth curves. Time of addition studies indicated that both virus-cell fusion and cell-cell fusion were inhibited by this compound. Selection of resistant viruses in vitro yielded two mutants with single point mutations in the F-protein: one upstream of the second heptad repeat motif and another within it (S398L and D486N). RSV titers, determined by quantitative RT-PCR, were reduced 10-fold in bronchoalveolar lavage fluid and in lung tissue of cotton rats treated once by inhalation with R170591 prior to RSV infection.

7.4. Fusion inhibitors for paramyxovirus

RFI-641, a compound that was derived by chemical optimization of the earlier described antiviral CL-387626, is a small molecule antiviral drug that selectively inhibits RSV (Wyde et al., 1998). The molecule is water-soluble and not orally bioavailable, but it proves to be efficacious when administered intranasally or by inhalation. The in vitro IC_{50} varies between 3 and 180 ng/ml for laboratory strains or clinical isolates of RSV subgroup A and B. Viral specificity and the large therapeutic window of RFI-641 (> 100 fold) indicate that the antiviral activity of the compound is not due to adverse effects on normal cells. Addition of RFI-641 to cell culture prior to adsorption reduces RSV yield 1000-fold at 48 h post infection (Wyde et al., 1998). Temperature shift experiments suggest that the RSV F protein is the target for RFI-641, and this observation is confirmed by the inhibitory effect that RFI-641 has on RSV B1 *cp*-52/2B5, a viable RSV mutant in which both the G and SH open reading frames are deleted. If RFI-641 is added to cell culture 5 h post infection, it inhibits syncytium formation indicating that fusion inhibition occurs both in the early and late phase of the infectious cycle. RFI-641-resistant viruses can be selected, albeit much less easily than amantadine resistant variants. Resistance to RFI-641 is acquired by point mutations solely in the F protein, mostly upstream of the second heptad repeat motif, but not by mutations in the G or SH protein. When administered prophylactically by the intranasal route, RFI-641 inhibits RSV replication in vivo, with mice and cotton rats exhibiting a 10–1000-fold reduction in RSV replication on day 5 post infection. In African green monkeys, RFI-641 reduces peak RSV titers not only when administered prophylactically but also when therapy is initiated at 24 h post infection

and continued for a total of 9 days. A nebulized form of RFI-641 has been shown to be active in monkeys. The preclinical profile of this drug supports its development for treatment and prophylaxis of RSV disease in pediatric, adult and geriatric populations. Phase 1 clinical trials confirmed that RFI-641 is a potent antiviral, and that the safety profile of this drug is encouraging.

7.5. Development of piconavirus 3C protease inhibitors

AG7088 is a potent, irreversible inhibitor of human rhinovirus (HRV) 3C protease, the enzyme that is responsible for the cleavage of the viral polyprotein into its functional protein subunits (Matthews et al., 1999). AG7088 was discovered by protein structure based rational drug design, and the compound exhibited activity against a large set of different HRVs. The 50% effective concentration (EC_{50}) ranges between 3 and 81 μM. Other piconaviruses such as coxsackievirus A21 or B3, enterovirus 70, and echovirus 11 are also sensitive to the compound. In a placebo controlled challenge study in which adult volunteers were infected with HRV39 and treated with AG7088 (8 μg per dose intranasally, 5 times per day), AG7088 reduced mean viral titers, as well as mucus weight, respiratory symptom score and total symptom score significantly. In this study viral titers were determined by culture and also by quantitative PCR (TaqMan) to exclude ex-vivo effects of AG7088 on the reduction of virus titers.

A phase II clinical trial with 868 subjects was conducted to determine the efficacy of AG7088 in naturally acquired piconavirus infections. Patients selected for the study had to present within 36 h of onset of symptoms and had to suffer from at least two mild or one moderate cold symptom. A five step symptom score was used to record severity of rhinorrhea, cough, sneezing, sore throat, chills, headache, and malaise. The treatment group was stratified into two or four daily doses of 8 μg AG7088 per dose intranasally, and the mean respiratory symptom score for days 1–5 was chosen as the primary endpoint. Only 29% of all enrolled patients were infected by picornaviruses. No significant difference in respiratory or total mean symptom score for days 1–5 was detected. However, the drug was well tolerated and safe. The lack of efficacy in this particular study might have been due to a lower than expected frequency of picornavirus infections. In a retrospective analysis, stratification for start of therapy within 24 h detected a trend to fewer respiratory symptoms and fewer total symptoms. There was a trend to earlier onset of relief, but the difference between treatment and placebo group again did not reach significance.

7.6. Pleconaril (P) treatment reduces the incidence of acute otitis media (OM) in children with viral respiratory illness: a pilot study

Pleconaril, an orally bioavailable picornavirus 3C protease inhibitor, has in vitro antiviral activity against 93% of all rhinoviruses. To evaluate the efficacy of pleconaril in preventing acute otitis media (AOM) in an outpatient population, a double-blind, placebo-controlled pilot study was conducted in children with viral respiratory illness. Eighty-seven children with a median age of 3 years and a history of 2–3 episodes of AOM were randomized to pleconaril treatment (5 mg/kg or 2.5 mg/kg) or placebo thrice daily for 7 days following presentation with upper respiratory symptoms. Picornavirus RNA was detected by RT-PCR in nasal samples in 51% of patients at baseline. The overall incidence of physician-diagnosed AOM during the 14 day follow-up were 18.8% (9/48), 12.5% (3/24), and 0% (0/15) in the placebo, low dose and high dose groups, respectively. Of the 87 children developing AOM, 86 tested positive for picornavirus RNA. Pleconaril treatment reduced the frequency of nasal symptoms by 29% in the high dose (5.0 mg/kg, $P = 0.006$) and 9% in the low dose (2.5 mg/kg, $P = 0.393$) group. It reduced frequency of systemic symptoms by 40% in the 5.0 mg/kg group ($P = 0.020$) and 26% in the 2.5 mg/kg group ($P = 0.096$). Pleconaril was well tolerated, and adverse events did not differ from the control group. These results encourage further expanded trials of pleconaril in children with viral respiratory infections.

7.7. Oral oseltamivir reduces influenza-related complications in all age groups

Influenza illness is associated with the development of secondary complications, often requiring antibiotic treatment or even hospitalization. Insurance data indicate that up to 64% of influenza related hospital admissions occur in 15–64 year old individuals, often without recognized underlying disorders. Influenza complications affect all age groups but the type of complication differs among them. While acute otitis media has been reported in over 20% of children with influenza, lower respiratory tract infections (LRTI) such as bronchitis and less often pneumonia, are the most common complication in adults. In recent years six phase III clinical trials were completed; two of them in pediatric populations, three in healthy adults and one in the elderly. The neuraminidase inhibitor oseltamivir (*O*) is already approved for the treatment of influenza in adults, and it was recently approved for treatment in children aged 1 year and older by the FDA. In six phase III trials, most of the patients were enrolled within 36 h of onset of symptoms, and oseltamivir was administered at 2 mg/kg twice daily for children and 75 mg twice daily for adults. Subjects with febrile (>37.8°C) influenza were randomized to a 5 day regimen of oseltamivir or placebo, and an important endpoint of all studies was the effect of oral oseltamivir (*O*) on the incidence of influenza-related complications requiring antibiotics. One thousand and twenty nine children between the age of 1 and 12 (mean age 6.4 years) were enrolled in the pediatric trials. In the adult and elderly populations, 953 patients with a mean age of 35 years and 736 elderly patients with a mean age of 73 years were enrolled. In the pediatric, adult and elderly trials 61, 64 and 65%, respectively, tested positive for influenza A or B by culture or fourfold increase in HAI titers. Physician-diagnosed secondary complications (bronchitis, pneumonia, LRTI, sinusitis or otitis media) requiring antibiotics were assessed in patients with confirmed influenza infection. Oseltamivir reduced the rate of complications compared with placebo by 40% in children ($P=65/235$, $O=36/217$; $P=0.005$), by 51% in adults ($P=23/309$, $O=11/301$; $P=0.05$), and by 28% in the elderly ($P=49/254$, $O=31/223$; $P=0.14$). In the pediatric population, AOM was the most common complication, followed by bronchitis, pneumonia and sinusitis. The frequency of AOM was reduced from 17% in the placebo group to 10% in the oseltamivir group, a significant 40% reduction. In adults and the elderly, bronchitis and sinusitis were the most common complications of influenza. The frequency of bronchitis in the elderly was reduced from 15 to 11%. Thus, oral oseltamivir reduced the incidence of secondary complications and associated antibiotic use in all age groups. When all age groups were combined, antibiotic use for any indication was also significantly reduced. Twelve patients in the placebo group versus four patients in the oseltamivir group required hospitalization for probable or possible influenza-related complications, suggesting a possible effect on hospitalization.

7.8. Neuraminidase inhibitors: clinical update

7.8.1. Oseltamivir

Oseltamivir was recently approved in the US for prophylaxis of influenza in adults and adolescents over 12 years of age after three successful phase three trials. One was conducted in healthy adults, one in a family setting with an infectious index case, and another evaluated seasonal prophylaxis in the elderly. The efficacy of oseltamivir in all three trials ranged between 70 and 90%. An application for the approval of oseltamivir for treatment of influenza in children over one year of age was recently approved by the FDA. In otherwise healthy children of 1–12 years, treatment with oseltamivir reduced the median time to freedom from illness by 1.5 days ($P=<0.0001$) and also reduced the relative risk of otitis media (AOM) by 40%. In 6–12 year old asthmatic children, oseltamivir treatment, started within 24 h of first symptoms, decreased the median illness duration by 1.7 days ($P=0.07$), and airway function was significantly improved (1 s forced expiratory volume on day 5 improved by 10.8% compared with baseline values among oseltamivir recipients and 4.7% among placebo recipients ($P=0.0147$)). The incidence of development of drug resistant

virus under therapy was evaluated in more than 1000 subjects. None of 542 subjects sampled on day 3 of treatment harbored resistant virus. On day 5, however, 4/451 subjects (1%) shed virus with a resistant phenotype. In children, the frequency of viral resistance was 4% (10/247). All of these subjects recovered normally despite this incidental finding. So far, resistant mutants have been less infectious than wild type virus; no evidence of transmission has been detected in an experimental infection model in ferrets.

The IMPACT study evaluated the benefit of early intervention in reducing the total time with symptoms starting from the time of first onset of fever. This reflects the total burden of illness from a patient's point of view. A total of 958 patients, 13–70 years of age were enrolled and treated within 48 h of the first onset of symptoms. Treatment was initiated within 12 h in 25% of those recruited. Using an accelerated time to failure model, earlier intervention was shown to strongly correlate with shorter duration of illness. The total duration of illness could be halved if treatment was started within 12 h of the onset of symptoms compared with intervention at 48 h.

7.8.2. Zanamivir

In phase III trials, Zanamivir reduced the duration of illness in adults over 12 years of age and in children between 5 and 12 years of age by 1.5 days (95% CI = 1–2.5 days). In high risk patients (525 subjects, 65% with asthma and 35% with chronic obstructive pulmonary disease) duration of illness amongst all influenza positive subjects was reduced by 1.5 days. Duration of illness was reduced in both vaccinated (1.25 day) and non-vaccinated (2 day) subjects. During therapy, pulmonary function improved slightly between days 1 and 5. Zanamivir was well tolerated, and there were no differences in adverse effects between treatment and placebo groups. With 9000 subjects studied in clinical trials, resistant mutants were not isolated by sampling on days 3 and 5. Efficacy for treatment of influenza B was assessed in 300 subjects and found to be similar to that of influenza A. A prophylaxis study in a family setting showed 79% protective efficacy. Finally, in a nursing home study, Zanamivir exhibited a 60% increased efficacy in preventing influenza disease compared with rimantadine.

7.8.3. RWJ-270201

RWJ-270201, an orally available once daily neuraminidase inhibitor that is active against influenza A and B in vitro and in animals, was evaluated in phase II trials of experimentally infected healthy adults. Therapy was started approximately 24 h post infection with influenza A/Texas H1N1 or influenza B/Yamagata. Viral load over time was the primary endpoint, and nasal wash titers were determined every 12 h on day 1–3 and daily thereafter. In the influenza A study, 90 subjects were enrolled into four treatment (100 to 400 mg per day) and one placebo arm. High dose treatment (400 mg once daily or 200 mg twice daily) reduced viral load by 68–73%, whereas 100 or 200 mg per day led to a reduction by 34%. Shedding was reduced by 1–2 days in the high dose groups, and the incidence of fever declined from 25% in placebo controls to less than 5% in all treatment groups combined. To evaluate efficacy against influenza B, 56 subjects were enrolled into two treatments (800 or 400 mg per day) and one placebo group. A 61% reduction of viral load was shown for the high dose treatment (800 mg per day). No evidence for the emergence of resistant virus was detected, and oral RWJ 270201 was well tolerated without excess gastrointestinal side effects.

8. Summary

World-wide, acute respiratory infections (ARI) are responsible for more than 4 million deaths per year in children under five years of age. Viral respiratory infections contribute significantly to this burden of disease, not only in children but also in adults. Although there is a myriad of viral respiratory pathogens, the number of virus families that cause significant burden of disease is limited. In children, the paramyxoviridae are the most important family of viruses, with respiratory syncytial virus (RSV) and the parainfluenza viruses (PIV) causing most disease, followed by adeno- and influenza viruses. In adults, influenza

A virus with its constantly changing antigenic composition surpasses other viral pathogens, but the impact of RSV and rhinoviruses are being increasingly appreciated. The epidemiology of respiratory virus infections very much depends on host and environmental factors. While adenovirus infections are a major concern in military training camps and in immunocompromised patients, they are not as important in the general adult population. The immunocompromised are not only at increased risk for viral respiratory disease but diagnosis and management are much more difficult in this group. Newer diagnostic methods based on antigen detection or viral genome amplification make rapid diagnosis possible and thereby affect clinical management of viral respiratory disease.

Vaccines are still the most powerful tool in preventing viral respiratory disease but for many important pathogens there is still no vaccine available. The inactivated influenza vaccine, with its antigenic composition changing annually, is still the mainstay of influenza prevention. Cold-adapted live attenuated influenza virus vaccines are on the horizon but are not yet approved by the FDA. Adenovirus vaccines have been used successfully in the military for many years, and the recent resurgence of large outbreaks of respiratory disease after discontinuation of vaccination emphasizes the importance of maintaining a preventive strategy. Live virus vaccine candidates against RSV and PIV, derived biologically or by reverse genetics, are currently in clinical trials, and there is hope that safe and efficient vaccines will become available within this decade.

New insight into virus-host and virus-bacteria interactions in viral respiratory illness may help us understand the complexity of these disease entities and allows a re-evaluation of management options. Diseases earlier thought of as purely bacterial, such as otitis media, might be more effectively prevented by viral vaccines than by bacterial vaccines. Populations at high risk for complications resulting from respiratory viral infections are now better defined and a more targeted prophylaxis is possible, be it passive prophylaxis against RSV disease with monoclonal antibody preparations or active prophylaxis with influenza- or adenovirus vaccines.

In the management of influenza virus disease, much has changed for the better. Neuraminidase inhibitors (NI) are now established as an effective intervention to decrease the severity and to shorten the duration of illness when therapy is initiated within 2 days of the onset of symptoms. Zanamivir and oseltamivir are now licensed in the US and elsewhere for adults and adolescents twelve years of age and older, and a license for use in children over one year of age has recently been obtained for oseltamivir. Both drugs are safe and well tolerated, and development of resistance occurs much less frequently than with older antivirals such as amantadine or rimantadine. Rhinovirus infections, the cause of most colds, may now be amenable to treatment with the experimental 3C protease inhibitors pleconaril (given orally) and AG7088 (given intranasally), and initial studies indicate that pleconaril treatment of colds in children might reduce their risk of developing otitis media. Antivirals effective against RSV and PIV are still in an early phase of development, but inhibitors of the viral fusion protein look promising.

Despite all progress, severe deficiencies remain in the preventative efforts. Our level of preparedness for an influenza pandemic does not seem much different from that 40 years ago. Streamlining of hospital infrastructure and just-in-time production of antibiotics and antivirals limit our ability to respond effectively should a pandemic strike. Although potential pandemic influenza viruses have been defined and novel techniques such as reverse genetics are being employed in vaccine development, the implementation of pandemic preparedness is still a daunting task.

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