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Emerging Respiratory Viruses: Challenges and Vaccine Strategies

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INTRODUCTION

Acute viral respiratory tract infections remain a leading cause of morbidity, mortality, and economic loss. Although often self-limiting in healthy adults, these infections are responsible for a substantial loss of productive time and are important factors in the illness and death of the very young, of immunocompromised individuals, and of elderly populations. In the last 10 years a number of novel human viral respiratory pathogens have been identified, leading to a heightened level of awareness and the development of measures to control them. The identification of novel viruses is both a result of the application of new, more sensitive techniques enabling the detection of viruses that have been circulating in the human population for years and the result of the recent introduction of viruses into the human population.

New and Newly Recognized Respiratory Viruses

SARS-CoV. In 2002, an unusually high incidence of atypical pneumonia was reported in southern China. The coordinated efforts of laboratories around the world identified a novel coronavirus, severe acute respiratory syndrome-associated coronavirus (SARS-CoV), as the causative agent of the outbreak (40, 103, 135, 146). The emergence of SARS-CoV in the human population is believed to be the result of zoonotic transmission (68). The outbreak lasted approximately 3 months, with 8,096 confirmed cases in 29 countries and with 774 deaths (http://www.who.int/csr/sars/country/table 2004_04_21/en/index.html). An additional four cases of community-acquired SARS-CoV infection were reported in 2003 to 2004, and two laboratory-acquired cases of SARS-CoV were reported in Taiwan and Singapore in April 2004 (176) (http://www.who.int/csr/don/2004_05_18a/en/index.html). Additional laboratory-acquired cases of SARS-CoV infection occurred in China and resulted in transmission to family contacts (http://www.cdc.gov/ncidod/sars/situation/may19.htm). No further cases of SARS-CoV infection have been reported, and the viral reservoir has not yet been conclusively identified.

Avian influenza virus. In 1997 an outbreak of avian influenza virus H5N1 occurred, resulting in the death of a 3-year-old boy in Hong Kong (27, 191). A total of 18 human cases of H5N1 influenza virus infection were confirmed in this outbreak, 6 of which proved to be fatal (8, 238). While avian influenza viruses are endemic in wild aquatic bird populations, and sporadic infections have occurred following direct inoculation with virus, this was the first identified introduction of avian influenza virus into the human population that resulted in disease. However, additional introductions of avian influenza viruses have occurred since. In 1998 to 1999, avian influenza viruses of the H9 subtype infected seven people in Hong Kong and China, causing nonfatal, influenza-like illness (70, 137). An additional human case of nonfatal H9 avian influenza virus infection was reported in 2003 (17), and the seroprevalence of H9 antibodies in the human population in southern China has been shown to be at least 2% (137); in 2003, 89 human cases of conjunctivitis, 13 cases of influenza virus-like illness, and 1 death, all linked to H7 avian influenza virus infection, occurred in The Netherlands, concurrently with an outbreak in poultry (50, 102), and additional cases of infection with H5 avian influenza viruses occurred again in Asia in 2003. H5N1 viruses spread to poultry throughout Asia and Europe and continue to spread to other countries. To date, more than 200 cases of human infection with H5N1 influenza virus have been confirmed in 10 countries, with a mortality rate of greater than 50% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_06_06/en/index.html). The H5N1 influenza virus outbreak continues to be of concern to the global public health community, as it could mark the beginning of the next influenza pandemic.

Human metapneumovirus. In 2001, the first metapneumovirus associated with infection and disease in humans was identified in The Netherlands (210). This human metapneumovirus, which is believed to account for up to 10% of respiratory infections in children under the age of 5 years, has since been identified in Australia, the United Kingdom, and North America (11, 129, 138, 140, 183, 210, 226). Analyses of archived specimens suggest that metapneumovirus has been circulating in the human population for at least 25 years (210, 226).

Human coronaviruses NL63 and HKU1. In 2004, two novel human coronaviruses (HCoVs) were identified in individuals with respiratory infections (47, 211). HCoV-NL63 has since been detected in individuals with typical features of acute respiratory infection in Europe, Japan, China, Australia, and North America, and HCoV-HKU1 was isolated from individuals with pneumonia (3, 5, 25, 122, 196, 208, 211, 228). Human CoVs, including the previously known HCoV-229E and HCoV-OC43, may account for up to 30% of respiratory infections in the general population (47, 84).

The generation of targeted vaccines and therapies is important for decreasing the morbidity and mortality associated with infection with respiratory viruses. The potential for reemergence of SARS-CoV from its as-yet-unidentified reservoir and the prevalence of HCoV-NL63, HCoV-HKU1, and human metapneumovirus in the population underline the need for development of vaccines targeting respiratory viruses. The rapid spread of avian influenza virus in avian populations throughout the world has raised awareness of this virus's potential to cause a pandemic and the urgency for the development of protective and/or preventive vaccine strategies. We can expect additional etiological agents of respiratory tract illnesses to be identified, as a proportion of these illnesses still cannot be attributed to known pathogens. Lessons learned from the development of vaccines against current respiratory viral pathogens should lend insight into development of vaccines against newly emerging agents.

In this paper we review the general principles of the response to newly identified virus infections and then focus on the development of vaccines against the recently emerged SARS-CoV and avian influenza viruses, as examples of pathogens that were or are significant public health concerns. SARS-CoV represents a newly discovered virus belonging to a virus family against which scientists have no experience developing human vaccines. In contrast, extensive work has been done on the development of human vaccines against human influenza viruses, and many of the same principles can be applied to vaccines targeting avian influenza viruses.

Response to Newly Identified Virus Infections

By following SARS from the recognition of the outbreak to the identification of SARS-CoV and, finally, to the development of vaccine strategies, we have gained insight into the processes involved in confronting a newly emerged viral pathogen. While avian influenza viruses have existed in avian populations for centuries, their recent introduction into the human population, with devastating results, provides another example of the viral threats that humans face. Before strategies for the development of targeted vaccines and antivirals against novel human respiratory pathogens can be initiated, several of the following steps must be taken.

Recognition of the disease or syndrome and establishment of case definition. Overlap in the clinical illnesses produced by respiratory viruses of different virus families can hamper the identification of novel, and potentially epidemic, viruses. "Influenza-like" symptoms are observed in infections with HCoVs, influenza viruses, human metapneumovirus, and respiratory syncytial virus, as well as other respiratory pathogens. These can also be the presenting symptoms in infections with nonrespiratory pathogens, including hepatitis B virus and Epstein-Barr virus. The clinical symptoms associated with human infection with SARS-CoV and H5N1 avian influenza virus include fever and sudden onset of "influenza-like" symptoms (including chills, malaise, and myalgia), followed by development of a cough and in some cases diarrhea (38, 135, 146; http://www.who.int/csr/resources/publications/WHO_CDS_CSR_ARO_2004_1/en/index.html; http://www.who.int/csr/disease/avian_influenza/guidelines/Guidelines_Clinical%20Management_H5N1_rev.pdf). Additionally, radiological evidence of consolidation in the lungs, lymphopenia, thrombocytopenia, and elevated liver enzyme levels have been reported in infections with both SARS-CoV and H5N1 avian influenza virus (7, 26, 135, 146). By using currently available diagnostic techniques, infection with known viral agents can be rapidly ruled out, as was the case with both the SARS-CoV and avian influenza virus outbreaks (40, 103, 135, 146). The initial case definitions for newly emerged diseases are generally broad in order to ensure that cases are not missed. During the SARS-CoV outbreak, the case definition included fever, cough, history of travel to affected areas, and close contact with an individual with suspected SARS (http://www.who.int/csr/sars/casedefinition/en/). A history of probable contact with other infected individuals or animals and the existence of other known infections in the area are important considerations during disease outbreaks. Human infections with H5N1 influenza virus are associated with direct contact with infected poultry, while infections with SARS-CoV were associated predominantly with contact with infected individuals (there was a high incidence in health care workers) or with infected animals (civet cats).

Identification of the causative agent. Once a case definition has been established, attempts to identify the causative agent of the disease can be initiated. Identification of novel viral pathogens can be hampered by the restricted species and tissue tropisms of the agent, as well as the inability to culture them using the current diagnostic tools and techniques. Coordinated activities of several laboratories using different approaches can speed up the identification of the etiological agent, as was demonstrated with the identification of SARS-CoV. Novel identification techniques, including random-primed PCR amplification strategies, which were used in the identification of human metapneumovirus and HCoV-NL63, and comprehensive DNA microarrays provide alternative means of identifying novel viral pathogens (47, 210, 213). Identification of a virus includes its classification as a member of a virus family. This classification is based on the size, genome organization, and genetic makeup of the virus (RNA or DNA genome) and is important for gaining an understanding of the biological properties of the virus. Human metapneumovirus, SARS-CoV, HCoV-NL63, and HCoV-HKU1 were identified as new members of previously established virus families based on virion morphology, genome organization, and genetic sequence (40, 47, 103, 135, 146, 210).

Biological properties of the virus. Elucidation of the biological properties of newly emerged viruses is vital in determining potential treatments and preventive strategies. Identification of sites of viral replication, kinetics of replication, and routes of entry and shedding provide scientists with an understanding of the viral life cycle and of steps that can be targeted by therapeutics and vaccines. Additionally, understanding the routes of entry and shedding aid in identification of potential modes of transmission and provide a means by which to decrease the risk of virus spread to naïve individuals. Identification of the cellular receptor for SARS-CoV and of the importance of the interaction between the surface glycoprotein and the receptor in mediating entry of the virus into cells has provided a potential target for therapeutic intervention and prevention (89, 111).

Diagnostic methods and surveillance. The development of methods to rapidly identify infected individuals is essential to monitor the spread of virus within susceptible populations. The development of reverse transcriptase (RT)-PCR based assays for the identification of SARS-CoV should help in future outbreaks to rapidly identify the infectious agent and allow for early intervention, with the caveat that the appropriate clinical specimens are collected and handled in a way that maintains the integrity of the viral RNA (144; http://www.who.int/csr/resources/publications/WHO_CDS_CSR_ARO_2004_1/en/index.html). The World Health Organization (WHO) recommends that at least two different techniques be used to accurately diagnose SARS-CoV infection. RT-PCR on two different clinical specimens (e.g., nasopharyngeal wash and stool) or on specimens collected at different times can decrease the risk of false-positive results, especially when coupled with serological assays (enzyme-linked immunosorbent assay [ELISA] or immunofluorescence assay) to measure a rise in antibody titer between acute- and convalescent-phase sera. RT-PCR-based assays have also proven to be effective in identifying infection with avian influenza viruses (http://www.who.int/csr/disease/avian_influenza/guidelines/Guidelines_Clinical%20Management_H5N1_rev.pdf). Surveillance is vital for monitoring the success of control strategies and should focus on identifying these agents in the human population, as well as in susceptible animal populations. In the case of avian influenza virus, surveillance in birds is essential and has led to interventions that have slowed the spread of the virus (168). Knowledge of the presence of virus within a susceptible animal population will enhance the speed at which human cases are identified and transmission prevented.

TABLE 1. Virus vaccine strategies

Vaccine strategy	Advantage(s)	Disadvantage(s)	Virus vaccine(s)
Inactivated (killed)	Stable formulations, safety in absence of viral replication, humoral immune response	Absence of strong cell-mediated immune response, multiple doses may be required, vaccine virus propagated to high titers (safety issue), potential adverse reactions (incomplete inactivation of infectious virus nucleic acids)	Influenza A and B viruses, poliovirus, hepatitis A virus, rabies virus
Live attenuated	Induce strong humoral and cell-mediated immune response	Low-level virus shedding may pose risk to immunocompromised persons, high mutation rate of RNA viruses (potential for reversion to wild type)	Measles virus, yellow fever virus, varicella-zoster virus, influenza A and B viruses
Subunit/expressed protein	Increased safety in absence of virus, large volumes can be produced rapidly	Multiple doses may be required, antigenic proteins and epitopes must be known	Hepatitis B virus
Vectored	Viral protein expressed in natural form		
DNA vaccines	Increased safety in absence of virus, relatively easy to design	Low immunogenicity in humans	

Therapeutic interventions. In the absence of vaccines and specific antiviral drugs, nonspecific therapeutic interventions are often implemented in an attempt to prevent severe morbidity and mortality. While this is difficult without a basic understanding of the pathogenesis of the disease, interventions are instituted based on observations of the clinical course of disease and complications, and it is often not possible to assess, or systematically compare, different therapeutic approaches during an outbreak. In the case of SARS, a majority of patients received antibiotics, steroids, and ribavirin; however, it is difficult to determine which strategy was beneficial and which may have been ineffective.

Correlates of protection. Ideally, the mechanisms of viral clearance and immune correlates of protection should be determined before vaccines are developed. However, as was the case with SARS-CoV, when a rapid response is needed in the face of an ongoing epidemic, vaccine development may be initiated in the absence of specific knowledge of the components of a protective immune response, by utilizing strategies developed for vaccines targeting other respiratory viruses. In the case of most respiratory viruses, both mucosal and serum antibody responses contribute to protection; however, it is important to determine if cell-mediated immunity (CMI) is also required for protection. Effective T-cell-based vaccines rely on the ability to induce immunological memory. Animal models have played a key role in the identification of immune correlates of protection.

Development of vaccines and antivirals. Strategies for the prevention and treatment of emerging viral pathogens generally begin after the steps outlined above have been completed. Table 1 summarizes different virus vaccine strategies. Ideally, an effective vaccine is one that induces and maintains significant concentrations of virus-specific antibodies in serum and at local points of viral entry (e.g., mucosal surfaces), as well as virus-specific T-cell immunity. A strong neutralizing antibody (NAb) response and a specific mucosal antibody response are desirable. The efficacy of a vaccine is often dependent on the biological characteristics of the virus, as well as the specific arms of the host immune system that provide protection against infection. Vaccination has proven to be effective in controlling polio, yellow fever, measles, and human influenza viruses and remains the most promising means by which to limit the spread and impact of emerging viral pathogens. However, in the case of potential global epidemic viral infections, vaccines must also be easy to administer and store because populations in developed and developing nations will need to be vaccinated. Additionally, in the case of a potential pandemic, production of a vaccine must be rapid if spread of the pathogen is to be limited. Finally, because the antigenic heterogeneity of circulating viruses cannot be predicted, the vaccine should provide cross-protection against potential variants of the virus.

SARS-CoV

Time Line of the Outbreak

In November 2002 an outbreak of atypical pneumonia was reported in Guangdong Province, southern China, and by the end of February 2003 the disease had spread to neighboring regions and distant countries, including Hong Kong, Taiwan, Singapore, and Canada (24) (Fig. 1). On 12 March 2005 the WHO issued a global alert, warning travelers and others to be aware of the signs and symptoms of SARS (http://www.who.int/csr/sars/archive/2003_03_15/en/). The rapid response of a coordinated group of laboratories and public health officials from around the world led to the identification of a novel CoV, SARS-CoV, as the causative agent of the outbreak (40, 103, 104, 135). Rapid case identification and isolation, contact tracing, with quarantine in some cases, and screening of travelers for signs of illness aided in a break in the human chains of transmission just 5 months after the initial global alert. However, the economic impact was devastating for the affected areas (177). The case fatality rate for SARS-CoV infection in 2003 was estimated to be 13.2% for individuals younger than 60 years and close to 50% for individuals 60 years and older (38). A total of 8,096 cases of SARS-CoV infection were reported in 29 countries, with a total of 774 deaths (http://www.who.int/csr/sars/country/table 2004_04_21/en/index.html).

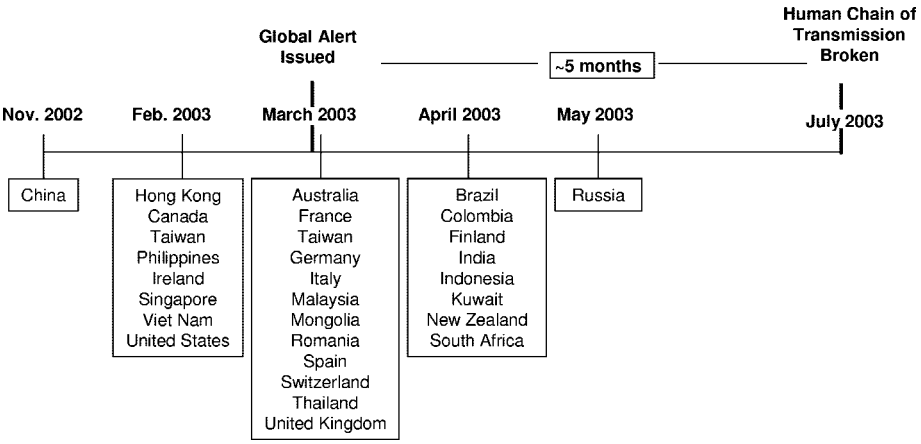

FIG. 1. Time line of the SARS-CoV outbreak. The countries in which cases of SARS-CoV were identified are listed.

Biological Properties of the Virus

Coronaviruses (order *Nidovirales*, family *Coronaviridae*) are enveloped, positive-stranded RNA viruses that utilize a unique and complicated mechanism of replication that results in a nested set of subgenomic RNAs (84, 107, 160). Coronaviruses are divided into three groups based on antigenic and genetic criteria (84, 107). While sequence analysis of several SARS-CoV isolates demonstrated that the genome of SARS-CoV has considerable nucleotide divergence from that of other known HCoVs, phylogenetic analysis indicates that it is distantly related to group 2 CoVs (i.e., HCoV OC43, mouse hepatitis virus, and bovine CoV) (103, 135, 174). Coronaviruses infect a wide range of species, including dogs, cats, pigs, mice, bats, and humans; however, most strains exhibit a narrow host range (84, 107, 143). Coronaviruses enter target cells via receptor-mediated endocytosis driven by the spike (S) glycoprotein, which protrudes from the surface of the virion. The S protein serves as the major viral attachment protein, critical to virus binding and fusion of the viral envelope (84). The receptor-S protein interaction is a major determinant of species specificity and tissue tropism for both group 1 and group 2 CoVs (37, 41, 84, 101, 105, 107, 227). Angiotensin-converting enzyme 2 and CD209L were identified as functional receptors for SARS-CoV; however, angiotensin-converting enzyme 2 serves as a more efficient receptor (89, 111, 214). Isolation of a virus that was genetically closely related to SARS-CoV from Himalayan palm civets and a raccoon dog in a live animal retail market in Shenzhen suggested that SARS-CoV was introduced into humans from an animal species (68). Sequence analysis of strains from a small, mild outbreak in Guangzhou in 2003 to 2004 further supports zoonotic transmission of SARS-CoV, as the viral genetic material from these human cases was more closely related to viral genetic material recovered from civets than to that from other human SARS-CoV isolates (176).

The evaluation of candidate vaccines requires animal models. Several animal species have been evaluated as potential animal models for SARS-CoV infection and associated disease. Nonhuman primates have been shown to be susceptible to SARS-CoV. However, although the virus replicates in the respiratory tracts of African green monkeys and rhesus and cynomolgous macaques, minimal to no clinical disease was observed (120, 148, 157). Clinical disease was observed in cynomolgous macaques inoculated with SARS-CoV in studies by Fouchier et al. and Kuiken et al.; however, these observations have not been uniformly reproducible (48, 104, 120, 157). Interestingly, liver enzyme elevation and thrombocytopenia, which were observed in many SARS-CoV-infected patients, were observed in nonhuman primates (120, 157). The common marmoset has also been shown to be susceptible to SARS-CoV infection with signs of clinical illness (63). Pneumonia and hepatitis were observed in inoculated animals, and mild colitis associated with watery diarrhea was observed, which resembled findings in some human cases (135).

Small animal models, which are less expensive and cumbersome to work with than nonhuman primate models, were also evaluated for susceptibility to SARS-CoV. BALB/c mice hold great promise as a model for SARS-CoV infection (192, 224). Although 6- to 8-week-old infected mice show minimal clinical disease, virus replicates to high titers in the lungs and nasal turbinates (192), and viral nucleic acid is detected in the lungs and intestines of these mice (224). Virus titers peak in the lungs by 2 to 3 days postinoculation (dpi), and virus is cleared by 5 to 7 dpi. Aged BALB/c mice (12 to 14 months) inoculated with SARS-CoV present with clinical signs of disease, including weight loss, hunching, and ruffled fur; the clinical symptoms resolve by 7 dpi, and no mortality is observed (153). High viral titers are detected in the lungs at 2 to 5 dpi, and virus is recovered from nasal turbinates and liver at 2 to 5 dpi. Golden Syrian hamsters are also susceptible to SARS-CoV infection, although they too show no overt clinical signs of disease upon infection (154). The kinetics of SARS-CoV replication in hamsters are similar to that observed in mice, with high titers of virus detected in the upper respiratory tract (URT) and lower respiratory tract (LRT) for up to 5 dpi and clearance from the lungs by 7 dpi. Histological evidence of pneumonitis, as indicated by multifocal infiltrates and areas of consolidation in the lungs, was observed in both aged BALB/c mice and hamsters following infection with SARS-CoV (153, 154). Similar changes, associated with alveolar damage, were observed in fatal human infections with SARS-CoV (135, 146). SARS-CoV infection and replication have also been observed in ferrets in the absence of clinical illness (34, 118). Viral RNA was de-

tected in the feces, blood, and pharyngeal swabs by RT-PCR for up to 5, 22, and 15 dpi, respectively, and infectious virus was recovered from pharyngeal swabs for up to 5 dpi. Other animal species shown to be susceptible to SARS-CoV in the absence of clinical disease include 129SvEv and C57BL/6 mice and cats (58, 83, 118). Although the animal models described above do not demonstrate the same degree of lethality or the full range of pathology observed in humans upon infection with SARS-CoV, they are very useful for the evaluation of vaccines, immunoprophylaxis, and immunotherapy strategies because the virus replicates to high titers in the respiratory tract and is associated with histopathological evidence of pneumonitis in some models. These animal models will also help us to better understand the pathogenesis and immune response to SARS-CoV infection.

Immune Correlates of Protection

Studies in the animal models discussed above have provided insight into the arms of the immune system involved in protection against SARS-CoV infection; however, there is still little known about the mechanisms of viral clearance. It is also unclear to what extent the disease observed in infected individuals is a result of the cytopathic effect of the virus and how much is a result of immunopathology. These questions must be answered if we are to determine the vaccine strategy that will provide the best protection, be it one directed towards the development of NAbs or towards the induction of specific cellular immune responses.

In several animal models, infection with SARS-CoV induces the production of NAbs which protect animals from subsequent virus challenge (120, 154, 192). Passive transfer of hyperimmune sera from previously infected mice was sufficient to protect naïve mice from subsequent virus challenge, demonstrating that NAbs are sufficient to restrict SARS-CoV replication (192). Monoclonal antibodies capable of neutralizing SARS-CoV by targeting the S protein, a potent inducer of NAb production, have been identified (62, 194, 195, 202, 209, 215). Elucidation of the epitopes recognized by these NAbs and the identification of the immunodominant epitopes/domains recognized in response to natural infection in SARS patients have demonstrated that the receptor binding domain of the S protein is a critical neutralization determinant (62, 75, 194, 195, 209). Prophylactic administration of MAbs generated by different methods, directed at the S protein of SARS-CoV, protected mice against subsequent virus challenge, suggesting that, in the absence of an approved vaccine, prophylactic administration of NAb may prevent or decrease the morbidity and mortality associated with SARS-CoV infection (62, 195, 202). Studies with hamsters established that the S protein is the only protective antigen; the other structural proteins did not contribute to protection (15).

Other correlates of protection are not as well understood. Kinetics of viral replication in mice deficient in natural killer T cells or in T and B lymphocytes were similar to those in normal mice, suggesting that viral clearance occurs through mechanisms independent of these immune components and likely relies on the innate immune system (58). The role of the innate immune system in viral clearance is supported by observations in mice with a targeted deficiency in Stat1. Viral replication in these mice was initially identical to that seen in normal mice, but it persisted for 5 to 22 dpi and progressed to diffuse interstitial pneumonia with progressive weight loss (83). Based on the observation that S protein-specific NAbs are detected in convalescent human sera and are sufficient to provide protection against SARS-CoV infection and replication in several animal models, most of the vaccine strategies published to date have targeted the generation of a S protein-specific humoral immune response.

Vaccines against SARS-CoV

Vaccines targeting several animal CoVs have been developed, and some have been demonstrated to be efficacious in preventing viral infection (158). However, a phenomenon of enhanced disease following vaccination has been observed in cats upon infection with feline infectious peritonitis virus following previous infection, vaccination, or passive transfer of antibody (142, 212, 222, 223). The phenomenon is not fully understood but is believed to be a result of enhanced uptake and spread of the virus through binding of virus-antibody immune complexes to Fc receptors on the surfaces of macrophages; low-titer (subneutralizing) antibodies directed against the S protein are mainly responsible (28, 130, 222). Although antibody enhancement appears to be limited to feline infectious peritonitis virus among CoVs, similar concerns have been raised with regard to SARS-CoV. Previously infected mice and hamsters are protected from subsequent infection with SARS-CoV in the absence of enhanced disease, and vaccine studies and passive immunoprophylaxis performed with mice and hamsters suggest that previous exposure and the presence of NAbs provide protection (154, 192). Exacerbated disease has not been observed in experimental infection of mice and hamsters with a range of NAb titers. There was no evidence of enhanced diseases in most studies, but the report of hepatitis in ferrets following challenge after administration of a modified vaccinia virus Ankara (MVA)-S protein vaccine and the in vitro observation of enhanced entry of pseudotyped virus in the presence of antibody induced against S proteins from some SARS-CoV strains but not others warrant further study (221, 235). SARS-CoV vaccine strategies that have been evaluated are listed in Table 2.

Inactivated whole-virus vaccines. Inactivated whole-virus SARS-CoV vaccines have been evaluated in mice. Takasuka et al. immunized BALB/c mice with a two-dose regimen of 10 μg of UV-inactivated SARS-CoV vaccine administered 7 weeks apart, in the presence or absence of alum (197). The initial dose induced high immunoglobulin G (IgG) antibody titers against SARS-CoV, and adjuvant increased these levels 10-fold. IgG levels were increased an additional 10-fold after the second vaccine dose. Serum antibodies specific for the viral nucleocapsid (N) and S proteins were detected and were maintained for at least 6 months, and activation of CD4 T cells was demonstrated, but vaccine efficacy against challenge was not reported (197). A formalin-inactivated whole-virus vaccine was also tested in mice following a four-dose regimen with each dose separated by 2 weeks (149). Serum NAbs were detected following both intranasal (i.n.) and subcutaneous (s.c.) inoculation, but efficacy of protection from subsequent viral challenge was not reported. Stadler et al. evaluated a β-proprio-

TABLE 2. SARS-CoV vaccines

Vaccine type[f]	Animal model	No. of doses/route/schedule	Notable outcomes (Ab/CMI/efficacy/other)	Reference(s)
Inactivated whole virus	BALB/c mice	2 doses/i.m. with 10 μg UV-inactivated virus in presence or absence of alum/0 and 7 wk	-5×10^3 IgG Abs at 7 wk, increased 10-fold with adjuvant and with boost; serum Abs specific to N and S proteins maintained at least 6 mo/activation of $CD4^+$ T cells/efficacy not determined	197
	BALB/c mice	4 doses/s.c. with 80 μg formalin-inactivated virus with alum/0, 4, 6, and 8 wk; 4 doses/i.n. with 50 μg inactivated virus/0, 2, 4, and 6 wk	After 2 injections had low to undetectable Ab levels by ELISA, after 4 injections all s.c.-vaccinated mice had high NAb titers (1:1,280)/efficacy not determined	149
	BALB/c mice	3 doses/i.m. with 5 μg β-propriolactone-inactivated virus with MF59 adjuvant/0, 2, and 4 wk	NAb titers of 1:91 detected after 2 doses/3 doses provided complete protection against challenge with 10^4 $TCID_{50}$[a] SARS-CoV	178
Subunit	BALB/c mice	3 doses/s.c. with 10 μg purified baculovirus-expressed soluble S1 protein and adjuvant/0, 28, and 56 days	Detected S protein-specific Ab, NAb detected after second inoculation/challenged i.n. with SARS-CoV on day 82, viral titers in URT were at or below the limit of detection/induced higher NAb and provided more complete protection than did inoculation with live virus	10
Vectored				
BHPIV3	African green monkeys	1 dose/i.n. and i.t.[b] with 10^6 $TCID_{50}$ live attenuated BHPIV3 expressing S protein	Induced serum NAbs (reciprocal titer of 3.9 $\log_2$)/challenged with 10^6 $TCID_{50}$ SARS-CoV-vaccinated monkeys protected upon challenge, no virus shed from URT or LRT	16
MVA	BALB/c mice	2 doses/i.n. or i.m. with 10^7 PFU MVA expressing full-length S protein/0 and 4 wk	S1 Ab detected at 4 wk, peaked at 6 wk, and began to decline; NAb detected after boost at 4 wk/mice challenged at 8 wk with 10^4 $TCID_{50}$ SARS-CoV, 2-dpi lung titers at or below the limit of detection; protection against viral replication in both URT and LRT	9, 21
MVA	Rhesus macaques	2 doses/i.m. with 5×10^6–5×10^7 $TCID_{50}$ MVA expressing full-length S protein/0 and 4 wk	Low NAb titer after first immunization, increase >100-fold after second immunization/ monkeys challenged 4 wk after boost, no virus isolated from lungs and no virus shedding detected, vaccine protective	21
MVA	Ferrets	2 doses/i.p.[c] and s.c. with 10^8 PFU MVA expressing full-length S protein/0 and 2 wk	NAb titer 7 days after boost (1,280–2,560), undetectable by 14 days/challenged 2 wk after boost, rapid immune memory response, virus shed in pharyngeal washes up to 29 days postchallenge, no protective effects observed/hepatitis observed following vaccination and challenge, viral antigen not detected in liver sections	221
VSV	BALB/c mice	1 dose/i.n. with 1.4×10^4 $TCID_{50}$ VSV expressing S protein	NAb titer 1:32/challenged mice 4 wk or 4 mo later with SARS-CoV, at 2 dpi lung viral titers at or below limit of detection, provided sustained protection	94
Rabies virus	BALB/c mice	1 dose/i.m. with 10^7 FFU[d] rabies virus expressing N or S protein	High NAb titers in S-vaccinated mice (1:161) but no NAbs in N-vaccinated mice/induced both humoral and cellular response/efficacy not determined	44
DNA vaccines	BALB/c mice	3 doses/i.m. with 25 μg DNA expressing S protein/0, 3, and 6 wk	Induced S-specific $CD4^+$ and $CD8^+$ T-cell responses and high NAb titers (1:50–1:150), presence of transmembrane domain induced higher NAb titers/challenged mice 30 days after last vaccination 10^6-fold reduction in viral load in lungs at 2 dpi, no evidence of productive viral replication/protection mediated by serum Ab	234
	C57BL/6 mice	3 doses/gene gun with 25 μg DNA expressing CRT-N/0, 2, and 4 wk	Induced higher levels of N-specific Abs and $CD8^+$ T cells than N alone/challenged mice with recombinant vaccinia virus expressing N, reduced viral titers in lungs of CRT-N-vaccinated mice	99
	BALB/c mice	3 doses/i.m. with 150 μg DNA expressing different S protein fragments/0, 3, and 6 wk	S1, S2, and N terminus of S1 induced SARS-CoV-specific Abs; only S1 and S2 in combination induced NAbs/efficacy not determined	241
	BALB/c mice	1 dose/electroporation with 30 μg DNA expressing different fragments of S protein	Induced both a humoral and CTL[e] response/efficacy not determined	74
	BALB/c mice	2 doses/i.m. with 50 μg DNA encoding S, M, or N/0 and 4 wk	All induced Abs. DNA-S induced highest Ab titers at 12 wk and lowest CTL response; DNA-M induced lowest Ab titers at 12 wk and highest CTL response/efficacy not determined	215
	BALB/c mice	2 doses/i.m. with 100 μg DNA encoding N, M, or E/0 and 2 wk	All induced IgG Abs. DNA-N induced highest Ab titers and CTL response/efficacy not determined	92
	BALB/c mice	3 doses/i.m. with 50 μg DNA encoding full-length S/0, 21, and 50 days; 2 doses/i.m. with 50 μg DNA encoding full-length S/0 and 21 days/ boost with 15 μg inactivated whole virus at 50 days; 2 doses/i.m. with 15 μg inactivated whole virus/0 and 28 days	All vaccines induced S-specific Abs and NAbs/whole-virus vaccination induced significantly higher Ab titers and higher cell-mediated immune response/ efficacy not determined	239

[a] $TCID_{50}$, 50% tissue culture infective doses.
[b] i.t., intratracheally.
[c] i.p., intraperitoneally.
[d] FFU, focus-forming units.
[e] CTL, cytotoxic T lymphocyte.
[f] BHPIV3, chimeric bovine/human parainfluenza virus 3; MVA, modified vaccinia virus Ankara; VSV, vesicular stomatitis virus.

lactone-inactivated whole-virus vaccine in the presence and absence of the adjuvant MF59, which has been approved for human use in Europe (178). Three doses of adjuvanted vaccine provided mice with complete protection against virus challenge, and NAb titers of 1:91 were detected after two doses. Thus, the immunogenicity and efficacy of inactivated SARS-CoV vaccines have been established in experimental animals, and one such vaccine is being evaluated in a clinical trial. However, the development of inactivated vaccines requires the propagation of high titers of infectious virus, which in the case of SARS-CoV requires biosafety level 3-enhanced precautions and is a safety concern for production. Additionally, incomplete inactivation of the vaccine virus presents a potential public health threat.

Live attenuated vaccines. To date, live attenuated vaccines for SARS-CoV have not been evaluated. However, systems have been developed to generate cDNAs encoding the genomes of CoVs, including SARS-CoV (2, 18, 236, 237). The panel of cDNAs spanning the entire CoV genome can be systematically and directionally assembled by in vitro ligation into a genome-length cDNA from which recombinant virus can be rescued (236). This system has been used for genetic analysis of SARS-CoV protein functions and will enable researchers to engineer specific attenuating mutations or modifications into the genome of the virus to develop live attenuated vaccines. While live attenuated vaccines targeting respiratory viruses, including influenza viruses and adenoviruses, have been approved for use in humans, the observation that infectious virus is shed in the feces of SARS-CoV-infected individuals raises concerns that a live attenuated SARS-CoV vaccine strain may also be shed in feces, with potential to spread to unvaccinated individuals (http://www.who.int/csr/resources/publications/WHO_CDS_CSR_ARO_2004_1/en/index.html) (40). Another concern is the risk of recombination of a live attenuated vaccine virus with wild-type CoV; however, there may be ways to engineer the genome of the vaccine virus to minimize this risk.

Subunit/expressed-protein vaccines. Bisht et al. reported on the development of a subunit vaccine consisting of a soluble baculovirus-expressed N-terminal fragment of the S protein (10). Mice received three 10-μg doses of the vaccine adjuvanted with monophosphoryl-lipid A and trehalose dicorynomycolate, a stable oil-in-water emulsion, or saponin adjuvant QS21 (demonstrated to enhance cellular and humoral immune responses in animals) subcutaneously at 0, 28, and 56 days and ELISA antibody recognizing full-length S protein, and high NAb titers were detected. The vaccine was highly effective in protection against subsequent intranasal challenge with SARS-CoV.

Vectored vaccines. Several groups have reported preclinical evaluation of vaccines utilizing other viruses as vectors for SARS-CoV proteins, including a chimeric parainfluenza virus, MVA, rabies virus, vesicular stomatitis virus (VSV), and adenovirus (9, 10, 15, 16, 21, 34, 44, 53, 94, 221, 240). Chimeric bovine/human parainfluenza virus 3 (BHPIV3), a live attenuated parainfluenza virus vaccine candidate, was utilized as a vector for the SARS-CoV structural proteins including S, N, matrix (M), and envelope (E), alone or in combination (15). Hamsters vaccinated with a single dose of S protein-expressing vaccine developed NAbs and were protected from challenge 28 days after vaccination, despite NAb titers two to fourfold lower than observed following infection with SARS-CoV (15). Protection was not observed in hamsters vaccinated with vaccine viruses expressing N, M, or E proteins, and there was no evidence of an additive effect with vaccines expressing combinations of proteins, indicating that S protein is the only significant protective antigen. Vaccination of African green monkeys with the S protein-expressing BHPIV3 vector induced SARS-CoV serum NAbs and protected the monkeys from subsequent challenge with SARS-CoV, as indicated by the lack of viral shedding from the upper and lower respiratory tracts (16). Modified vaccinia virus Ankara expressing full-length SARS-CoV S protein induced potent SARS-CoV NAbs in mice, ferrets, and rhesus macaques (9, 21, 221). Inoculation of BALB/c mice intranasally or intramuscularly (i.m.) with two doses of MVA expressing S protein at 0 and 4 weeks provided protection from subsequent virus challenge at 8 weeks (9). A similar vaccine also protected rhesus macaques from subsequent challenge, with virus detected in nasopharyngeal washes of only one of four animals at 2 dpi and an inability to isolate virus from the lungs (21). In both studies with MVA expressing SARS-CoV S protein, NAb titers were significantly increased after the second dose of vaccine. However, vaccination of ferrets with two doses of an MVA expressing SARS-CoV S protein failed to induce high-titer NAbs and failed to protect ferrets from SARS-CoV infection and replication, possibly due to the differences in the vaccine itself or to levels of MVA replication in this species (221). Vaccination of mice with rabies virus expressing the SARS-CoV S or N protein was also evaluated as a vaccine strategy (44). Spike protein-expressing rabies virus vaccine induced high levels of NAbs, while the N-expressing vaccine did not, further supporting the role of S as the primary viral antigen capable of inducing NAbs. An attenuated VSV-based vaccine expressing the SARS-CoV S protein completely protected mice against subsequent virus challenge at 4 weeks and 4 months postvaccination (94). A single vaccine dose induced NAb titers higher than those observed following live SARS-CoV infection (1:32 and 1:12, respectively), and transfer of serum from vaccinated mice to naïve mice protected the naïve mice from subsequent virus challenge. Vaccination with adenovirus-based vectors expressing the S, M, and N proteins of SARS-CoV at 0 and 28 days induced a strong NAb response as well as N-specific T-cell responses in vaccinated rhesus macaques; however, subsequent virus challenge was not performed (53). Studies with vectored vaccines further demonstrate that induction of S protein-specific NAbs is sufficient to confer protection.

DNA vaccines. DNA vaccines have demonstrated strong induction of immune responses to viral pathogens in animal models, specifically in mice; however, clinical data on DNA vaccines in human subjects are limited. DNA vaccines encoding the S, N, M, and E proteins of SARS-CoV have been evaluated in mice (74, 92, 99, 216, 234, 241, 242, 244). Vaccination with S-, M-, and N-encoding DNA vaccines induced both humoral and cellular immune responses, with some variation in the relative levels of induction (92, 216). Yang et al. evaluated DNA vaccines encoding different fragments of the SARS-CoV S protein by vaccinating BALB/c mice with three doses intramuscularly at 0, 3, and 6 weeks (234). Increases in SARS-CoV S protein-specific $CD4^+$ T-cell immune responses

and $CD8^+$ cellular immunity were accompanied by substantial NAb titers (1:50 to 1:150). Interestingly, DNA vaccine constructs with the transmembrane domain of S protein induced substantially higher NAb titers than did S lacking the transmembrane domain. Vaccinated mice challenged with SARS-CoV 30 days after the third vaccine dose had a 10^6-fold reduction in viral titers in the lungs and no evidence of productive viral replication. Depletion of $CD4^+$, $CD8^+$, and $CD90^+$ T cells did not affect vaccine efficacy, and passive transfer of antibodies, but not of T cells, transferred protection to naïve mice, indicating that this vaccine mediated protection through NAb (234). Kim et al. evaluated the immunogenicity of a DNA vaccine encoding the N protein linked to calreticulin (CRT-N), an endoplasmic reticulum protein that reportedly enhances antigen-specific immune responses (99). Vaccination of C57BL/6 mice with three doses of CRT-N vaccine administered at 2-week intervals induced higher levels of both N-specific antibodies and $CD8^+$ T cells than vaccination with N-encoding DNA alone. The CRT-N vaccine was shown to protect mice from subsequent challenge with a recombinant vaccinia virus expressing the SARS-CoV N protein. Combination vaccines have also been evaluated for their ability to augment immune responses to SARS-CoV (229, 239). Administration of two doses of a DNA vaccine encoding the S protein, followed by immunization with inactivated whole virus, was shown to be more immunogenic in mice than either vaccine type alone (239). The combination vaccine induced both high humoral and cell-mediated immune responses. High NAb titers were also observed in mice vaccinated with a combination of S DNA vaccines and S peptide generated in *Escherichia coli* (229). Combination vaccines may enhance the efficacy of DNA vaccine candidates.

The SARS-CoV vaccine strategies reported to date demonstrate that S protein-specific NAbs alone are sufficient to provide protection against viral challenge. While SARS-CoV has not yet reemerged, its unknown reservoir leaves open the possibility that it, or a related virus, will again infect the human population. The development of vaccines targeting this virus will help, in the event of its reemergence, to potentially stop its spread before it wreaks the social and economic havoc caused by the previous outbreak. Furthermore, lessons learned from the generation of these vaccines may aid in the development of future vaccines against known and newly identified CoVs.

AVIAN INFLUENZA VIRUS

Biological Properties of Influenza Viruses

Influenza virus is a member of the *Orthomyxoviridae* family, which consists of four genera: *Influenza A virus*, *Influenza B virus*, *Influenza C virus*, and *Thogotovirus*. Influenza B and C viruses infect humans, while influenza A viruses, which are the focus of this review, infect humans, avian species, swine, and other mammalian species (232). Influenza A virions are enveloped and contain eight segments of single-stranded, negative-sense RNA, which encode 11 proteins. Embedded in the viral envelope are the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are the major antigenic determinants of influenza viruses (232). HA mediates binding of influenza virus to cells by interaction with sialic acid-containing receptors on the target cell surface. HA is synthesized as a single polypeptide (HA_0) but is subsequently cleaved by proteases into two disulfide-linked chains (HA_1 and HA_2), leading to exposure of a hydrophobic fusion peptide, which mediates fusion of the viral envelope with the target cell membrane (reviewed in reference 173). Cleavage of HA is required for viral infectivity and is a critical determinant of viral pathogenicity (12, 164). There are currently 16 known HA antigenic subtypes (H1 to -16), all of which are found in aquatic birds; however, sustained epidemics in humans have been limited to the H1, H2, and H3 subtypes (49, 232). NA is a sialidase that cleaves sialic acid residues from the cell surface and is critical for release of virus from infected cells (232). Blocking of NA activity by the use of neuraminidase inhibitors prevents release of new virions from infected cells (reviewed in reference 123). Nine NA subtypes have been identified (N1 to -9), of which the N1 and N2 subtypes have been found in human influenza viruses. The HA and NA surface glycoproteins are the major protective antigens of influenza viruses. The antigenicity of influenza viruses changes gradually over time (antigenic drift) as a result of accumulation of point mutations, which result from immune pressure, and error-prone RNA virus polymerases. Rapid and more drastic changes in influenza viruses can also occur as a result of introduction of a virus bearing a novel HA into humans. This can occur when an animal or avian influenza virus directly infects humans or can result from coinfection with two influenza viruses during which progeny viruses can derive gene segments from both, resulting in the generation of novel reassortant viruses. Antigenic shift results in the generation of viruses containing HAs to which the majority of the human population is naïve and can cause an influenza pandemic if the virus spreads efficiently from person-to-person. Although recombination events are rare, there are reports of recombination between the HA and other viral gene segments and possibly with cellular DNA among H7 viruses (186).

History of Influenza Pandemics

Natural reservoir of influenza A viruses. Avian species, specifically shorebirds and waterfowl, are the natural reservoir of influenza type A viruses and play an important role in the ecology of the viruses (220). Viruses of all 16 HA and 9 NA subtypes have been isolated from waterfowl, in which infections are generally asymptomatic and limited to the intestinal tract (220, 232). Virus is excreted in the feces of waterfowl and can survive for several weeks in water, promoting transmission to uninfected birds via the fecal-oral route (218). As wild ducks migrate along flyways, fecal excretion of virus results in spread to other wild and domestic avian species, including turkeys and chickens (72, 88). Influenza A viruses can also be transmitted from the aquatic bird reservoir to mammalian species, including humans, seals, whales, horses, pigs, and domestic poultry (56, 66, 71, 77, 86). Ecological studies suggest that all mammalian influenza A viruses are derived from the avian influenza virus reservoir (218). Although influenza viruses are continually adapting to mammalian hosts, they appear to be in evolutionary stasis in aquatic birds (6). Avian and human viruses preferentially bind sialic acid molecules with specific oligosaccharide side chains with α2,3 and α2,6 linkages, respectively. Receptor specificity was thought to be an important determi-

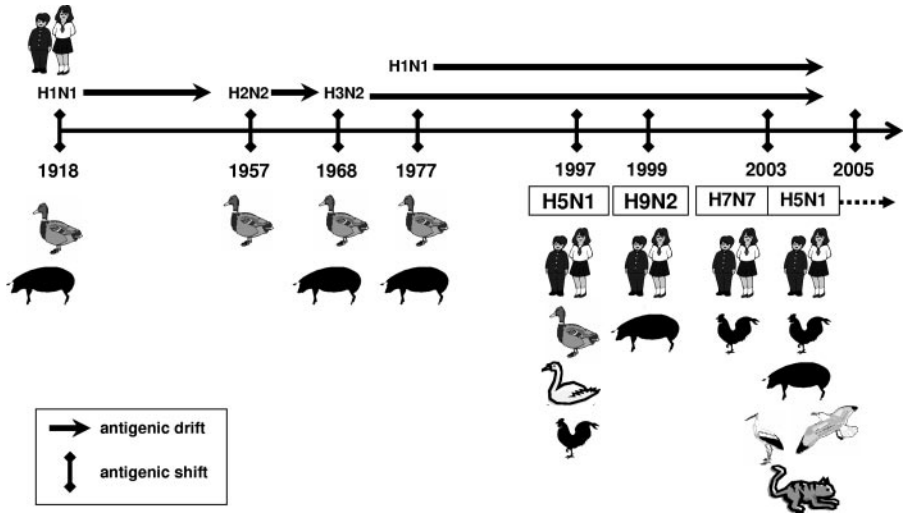

FIG. 2. Time line of influenza pandemics and outbreaks. Human pandemics are depicted above the time line, and animal infections and avian influenza virus outbreaks in humans are depicted below the time line.

nant of viral host range, as demonstrated by the fact that reports of experimental human infections with avian influenza viruses were rare. However, reports of H5N1, H9N2, and H7N7 virus infections in humans demonstrate that receptor specificity does not present an absolute restriction. Also, recent studies with differentiated human airway epithelial cells have demonstrated that cells bearing α2,3- and α2,6-linked sialic acids are present in human respiratory epithelium (119). Pigs are susceptible to both avian and human influenza viruses, due to the presence of sialic acid molecules with both α2,3 and α2,6 linkages to galactose on their tracheal epithelium, and could potentially serve as mixing vessels for the reassortment of avian and human viruses (87). Human-swine reassortant viruses have been detected in pigs in Japan, Europe, and the United States, demonstrating that genetic reassortment can occur in nature between influenza A viruses in pigs, but these resulting reassortants have not yet resulted in an epidemic virus strain in humans (162). While swine influenza viruses have been sporadically transmitted to humans, none of these incidents has resulted in an epidemic disease in humans (35, 39, 54, 55, 60, 78, 81, 98, 155, 193, 201, 225).

Previous pandemics. In temperate climates, epidemics of influenza occur every year, beginning in late winter and lasting 1 to 2 months (13). Influenza virus infection leads to the hospitalization of more than 100,000 people per year in the United States, killing more than 30,000 people in an average year (171, 199). Pandemics have occurred at irregular intervals every 10 to 50 years for the last several centuries and have resulted in a far greater number of deaths (33, 232). In the last century, three influenza virus pandemics occurred: the 1918 "Spanish influenza," the 1957 "Asian influenza," and the 1968 "Hong Kong" influenza (Fig. 2). The 1918 to 1919 influenza pandemic emerged swiftly in 1918, with outbreaks occurring almost simultaneously in North America, Europe, and Africa (reviewed in reference 151). Although most cases of illness were mild, 25 to 40% of the population in communities experienced morbidity, and a high percentage of patients developed severe complications (i.e., pneumonia). Interestingly, in contrast to subsequent influenza epidemics and pandemics, in which persons younger than 65 years of age accounted for 36 to 48% of excess influenza-related deaths, more than 99% of influenza-related deaths in 1918 were observed in persons younger than 65 years of age (reviewed in references 151 and 170). The most severely affected group were individuals between the ages of 20 and 40 years (170). Isolation and sequence analysis of RNA from fixed and frozen lung tissues of victims of the 1918 pandemic and the recent characterization of reconstructed 1918 pandemic virus suggest that the 1918 virus was an avian-like virus that adapted to humans (150, 198, 207). H1N1 influenza viruses circulated in the human population for 39 years, until the emergence of a new pandemic virus containing two novel surface glycoproteins (H2 and N2). Reassortment between the circulating human H1N1 virus and an avian H2N2 virus resulted in the introduction of novel HA, NA, and PB1 gene segments from the avian virus into the background of the H1N1 virus (57, 96, 163). The novel H2N2 influenza virus rapidly spread throughout the human population, displacing the H1N1 viruses (161). Because there was little or no preexisting immune protection from this subtype in the human population, the emergence of H2N2 influenza viruses in 1957 resulted in a pandemic. During the H2N2 pandemic, there were approximately 70,000 excess deaths in the United States, with the highest attack rates (~50%) occurring in children between the ages of 5 and 19 (59, 113). In July of 1968, another pandemic influenza virus emerged, this time in Hong Kong. The 1968 influenza virus was the product of reassortment between the circulating H2N2 human influenza virus and an avian H3 virus from which the HA and PB1 gene segments were derived (45, 57, 96, 163). The H3N2 influenza virus killed more than 30,000 people in the United States, with attack rates highest (~40%) among children between the ages of 10 and 14 years (113). This pandemic may have been less severe than previous pandemics because of the presence of antibodies against N2 in the human population (113). H3N2 viruses continue to circulate in the human population today. In 1977 an H1N1 influenza virus, closely related to viruses that had been circulating in and

around 1950, reappeared in the human population, but it did not result in a true pandemic because a large portion of the population had been previously exposed to H1N1 viruses (126). However, H1N1 viruses became reestablished, and H1N1 and H3N2 viruses continue to cocirculate in the human population with influenza B viruses.

Avian influenza virus infections in humans. (i) Known cases of avian influenza virus infection in humans. Until recently, it was believed that differences in receptor specificity provided a barrier against human infection by avian influenza viruses. The recently documented direct transmission of avian H5N1, H7N7, and H9N2 influenza viruses from birds to humans indicates that receptor specificity cannot fully restrict virus infection and that an intermediate host is not necessarily required for transmission to humans (Fig. 2).

(a) H5N1 avian influenza. In May 1997, an H5N1 influenza virus was isolated from the tracheal aspirate of a 3-year-old boy who died of respiratory failure in Hong Kong (27, 36, 191). This was the first reported avian influenza virus infection shown to cause severe respiratory disease in humans. Seventeen additional human cases of H5N1 influenza virus infection were identified by virus isolation and/or serology between November 1997 and January 1998, and six deaths occurred (8, 238). Outbreaks of highly pathogenic H5N1 disease associated with 75% mortality occurred concurrently on chicken farms in Hong Kong (169). The viruses that caused the human and chicken outbreaks were found to be genetically and antigenically closely related, suggesting that direct transmission from birds to humans had occurred (169, 187, 243). There was limited evidence of human-to-human transmission, and most of the cases were caused by independent transmission of virus to humans from birds in poultry markets or elsewhere (14, 95, 124, 243). The 1997 H5N1 influenza virus was a reassortant between an H5N1 virus that circulated in geese (A/goose/Guangdong/1/96) and an H6N1 virus (A/teal/HK/W312/97) present in ducks and/or an H9N2 quail virus (A/quail/HK/G1/97) (67, 112, 233). This highly pathogenic H5N1 virus was eradicated through mass culling of infected poultry; however, the putative precursor viruses continued to circulate in the avian population (65). In 2001, another avian outbreak of H5N1 influenza virus occurred in Hong Kong retail live poultry markets, caused by viruses related to H5N1 influenza viruses isolated from aquatic birds in Hong Kong during the previous 2 years (64). In 2003, three members of a Hong Kong family visiting Fujian province became ill, and two of them died from respiratory failure (136). H5N1 influenza viruses were isolated from the father who died and the son who recovered (136). In late 2003 to early 2004, reports of large outbreaks of highly pathogenic H5N1 influenza virus infection were reported in poultry throughout South Korea, Japan, Indonesia, Viet Nam, Thailand, Laos, Cambodia, and China. Ten individuals infected with H5N1 influenza virus, and with a clear history of exposure to sick poultry, were identified in Vietnam (203). From December 2003 to 6 June 2006, 225 confirmed cases and 128 deaths were reported in 10 countries (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_06_06/en/index.html). The virus continues to spread among avian species and has now been detected in 33 countries, including Croatia, Kazakhstan, Turkey, Mongolia, the Philippines, Romania, Japan, Korea, Laos, Malaysia, and Russia (Fig. 3) (http://www.oie.int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm). With a demonstrated ability to infect humans, the rapid spread of highly pathogenic H5N1 influenza viruses in birds has heightened concerns about the potential for an influenza pandemic.

(b) H9N2 avian influenza virus. In Hong Kong in March 1999, H9N2 influenza viruses were isolated from two children (1 and 4 years old) presenting with mild and self-limited febrile illness (137). Person-to-person transmission was not observed; however, NAbs to H9N2 influenza viruses were detected in the sera of volunteer blood donors, suggesting that other human infections with H9N2 influenza viruses have occurred in Hong Kong (137). A report in the Chinese language literature describes five additional human infections with H9N2 viruses (70). In 2003, an H9N2 influenza virus was isolated from a 5-year-old boy presenting with respiratory symptoms and hospitalized in Hong Kong (17). To date, H9N2 influenza virus infection in humans has resulted in mild, nonlethal illness. However, widespread infection of poultry in Asia with H9N2 influenza viruses, coupled with the cocirculation of H9N2 and H3N2 influenza viruses in pigs in southeastern China, could result in the generation of potentially pandemic reassortant strains of influenza virus with virulence that cannot be predicted (65, 134).

(c) H7N7 avian influenza virus. Outbreaks of highly pathogenic avian influenza virus H7N7 infection were reported in birds beginning in 1975 (1, 139, 186). Isolated cases of human infections with this subtype of influenza virus transmitted from birds and harbor seals have been reported (4, 106). In The Netherlands in February 2003, an outbreak of highly pathogenic avian influenza virus H7N7 infection emerged, which spread to poultry in Germany and Belgium (50, 102). Over the next three months, 89 cases of human H7N7 influenza virus infection were confirmed in agricultural workers who were involved in culling infected poultry, with evidence of transmission between poultry workers and their families (50, 102). Of the 89 confirmed cases, 78 developed conjunctivitis, 5 developed conjunctivitis and influenza-like illness, 2 developed influenza-like illness, 4 did not fit the case definition, and 1 veterinarian died (50, 102). Recent serological studies indicate that additional human infections with H7N7 avian influenza virus occurred during this outbreak in persons exposed to poultry (49%) and in persons exposed to H7-infected individuals (64%) (121).

(ii) Virus subtypes of greatest concern. To date, the transmission of avian influenza viruses to humans that has resulted in disease has been limited to the H5, H7, and H9 subtypes. However, serological evidence suggests that poultry and live bird market workers in Asia have been exposed to several avian influenza virus subtypes (167). The fact that H6N1 viruses circulating in Asia and the H5N1 viruses that have infected humans share seven gene segments suggests that they may have the potential to infect humans (23). Although severe disease in humans has been observed only following direct infection with avian influenza viruses of the H5 and H7 subtypes, the fact that the H2N2 and H3N2 virus pandemics, with attendant morbidity and mortality, were caused by reassortment between avian and human viruses suggests that mutations or reassortants of the other avian influenza viruses could also potentially generate pandemic influenza virus strains.

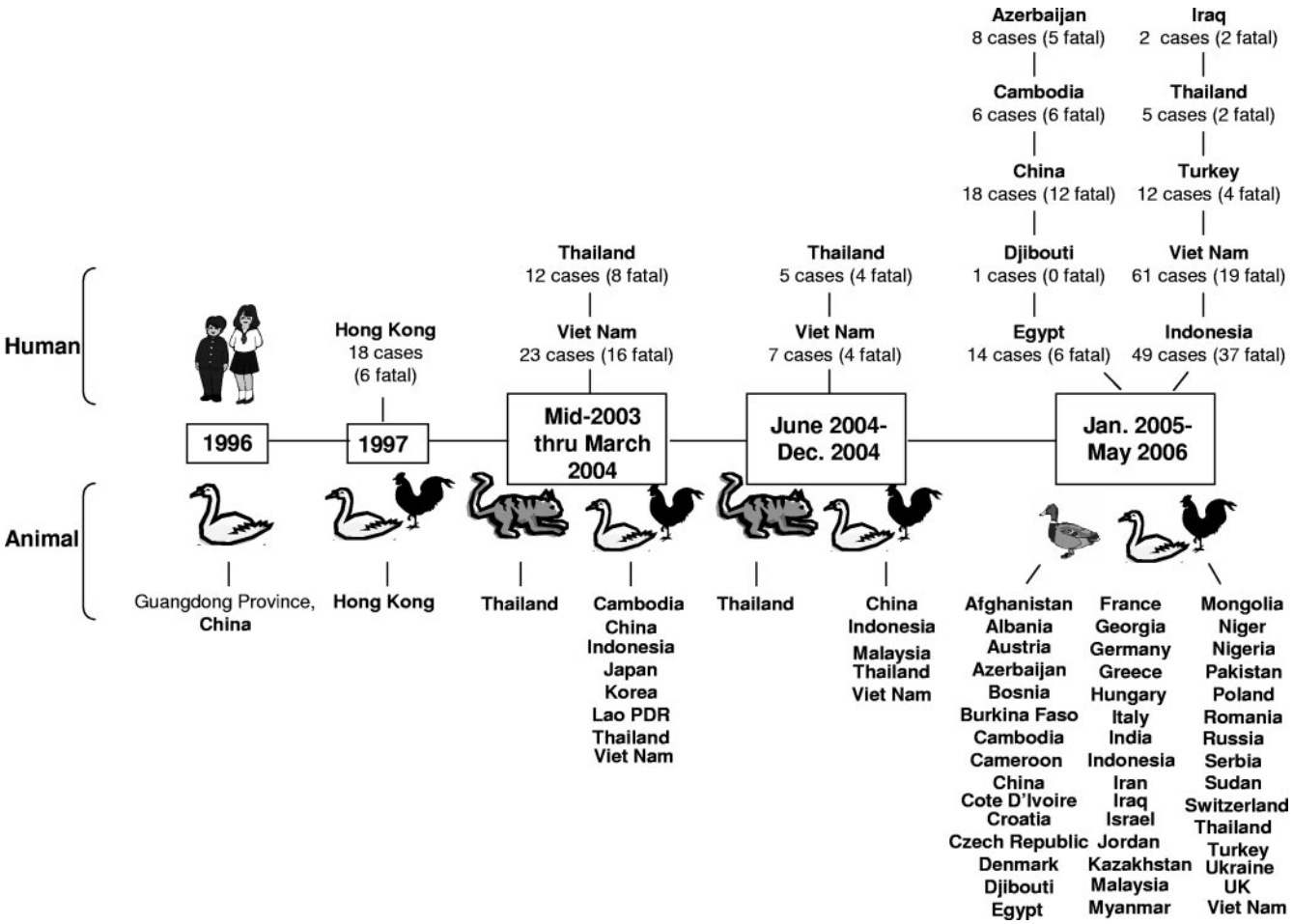

FIG. 3. Time line of avian influenza H5N1 outbreak and spread. Human infections are reported above the time line, and nonhuman infections are reported below the time line.

(iii) Pathogenesis. The virulence of avian influenza viruses has been well studied in avian species but in mammals is not as well understood. Here we focus on the pathogenesis of the H5N1 influenza viruses and how this information can aid in the development of vaccine strategies. Variability in the severity of disease caused by H5N1 influenza virus isolates has been observed in ferrets and mice and may provide insight into the determinants of pathogenicity (42, 52, 61, 115). However, both BALB/c mice and ferrets can be infected intranasally, with systemic spread of some of the viruses, lethality, and histopathological presentation similar to that observed in some fatal human infections (52, 61, 69, 115, 169, 245). These animal models have been used to study pathogenesis of H5N1 disease and for the evaluation of antivirals and vaccines. Highly pathogenic H5N1 influenza viruses have continued to evolve since their emergence in 1997, with changes in antigenicity (85, 172) and internal gene constellation, expanded host range in avian species (141, 185), and enhanced pathogenicity in experimentally infected mice and ferrets (61, 245). Virulence of influenza viruses is a polygenic trait and has been attributed to several viral genes; however, the HA protein is a major virulence factor in poultry (73, 97, 156, 219). The HA of low-pathogenicity influenza viruses is cleaved by proteases that are limited to the intestinal tracts of avian species and to the respiratory tracts of mammalian species. In contrast, highly pathogenic H5 and H7 viruses have acquired multiple basic amino acids adjacent to the HA cleavage site, allowing for cleavage by ubiquitous proteases of the subtilisin family and resulting in systemic spread of the virus (164, 182, 230). Other amino acid changes associated with changes in viral pathogenicity have been identified: a specific amino acid substitution in the polymerase subunit PB2 is associated with enhanced viral replication and lethal infection in mice (73), and an amino acid substitution within the NS1 protein of the H5N1 1997 influenza virus correlates with resistance to inhibition by interferons and tumor necrosis factor alpha in vitro (165). The innate immune response induced by H5N1 influenza virus infection may also contribute to its pathogenesis. H5N1 influenza viruses induce high levels of cytokine production in human macrophages, and individuals infected with the 1997 H5N1 virus exhibited elevated interleukin-6, tumor necrosis factor alpha, and gamma interferon levels, findings that are consistent with cytokine dysregulation, which could exacerbate tissue damage (22, 165, 200).

(iv) Immune correlates of protection. The immune correlates of protection are fairly well described for human influenza vi-

TABLE 3. H9N2 avian influenza virus vaccines

Vaccine type	Test system	No. of doses/route/vaccine strain/schedule	Notable outcomes (Ab/CMI/efficacy/other)	Reference
Inactivated whole virus	BALB/c mice	1 dose/i.m. with 10 μg formalin-inactivated A/Hong Kong/1073/99 (G1) or Ck/G9 (G9) ± alum	Both vaccines induced HI Ab titers of ≥1:40 to homologous virus in the absence of alum, HI Ab titers increased 6–12-fold in presence of alum; G1 vaccine induced Ab titers against both homologous and heterologous virus, G9 vaccine induced Ab titers only against G9 viruses/challenged i.n. 3 mo postvaccination, G1 vaccine provided complete protection against both homologous and heterologous challenge, G9 vaccine provided complete protection against homologous challenge and partial protection against heterologous challenge	114
	Humans	2 doses/i.m. with 7.5, 3.8, or 1.9 μg A/Hong Kong/1073/99 (H9N2) + mineral adjuvant/0 and 3 wk	After first dose, protective Ab titers were not detected; after second dose, protective Ab titers of ≥1:40 were detected	76
	Hamsters	2 doses/i.p.[a] with 15 μg formalin-inactivated A/Hong Kong/1073/99 ± alum/0 and 3 wk	HI and NAb titers were induced in vaccinated animals/challenged i.n. with homologous virus 4 wk after final vaccination, and all animals immunized with vaccine alone were completely protected against challenge (no virus detected in lungs 2 dpi); half of animals immunized with vaccine + alum were not protected	159
	BALB/c mice	2 doses/i.m. with 10 μg formalin-inactivated G9/PR8 reassortant ± alum/0 and 2 wk	After 1 dose, high HI Ab titers (≥320) against G9 virus were detected, no HI Ab against heterologous G1 virus; after second dose, HI Ab titers of 1:80 detected against G1 virus, increased fourfold with alum/challenged i.n. with G9 or G1-like virus, complete protection against homologous G9 and heterologous G1-like virus after 2 doses ± alum	20
	Humans	2 doses/i.m. with 7.5, 15, or 30 μg A/Hong Kong/1073/99 whole-virus or subunit vaccine/0 and 3 wk	HI geometric mean Ab titers did not differ between any doses on days 21 and 42 for the whole-virus vaccine or the subunit vaccine, HI and NAb titers were significantly lower in individuals born after 1968; 2 doses of either vaccine induced protective Ab titers in people born before 1968, and one dose of either vaccine induced protective Ab titers in people born before 1968/both vaccines were well tolerated without adverse side effects, subunit vaccine was better tolerated than whole-virus vaccine	181
Live attenuated virus	BALB/c mice	1 dose/i.n. with 10^5 $TCID_{50}$[b] of G9-A/AA/6/60 *ca* reassortant	Elicited HI Ab titers against homologous virus (HI = 320) and heterologous virus (HI = 40) 4 wk postvaccination/challenged i.n. with 10^5 $TCID_{50}$ of either homologous or heterologous virus, G9/AAca virus vaccine protected mice from virus challenge (no virus detected in URT or LRT)	19

[a] i.p., intraperitoneally.
[b] $TCID_{50}$, 50% tissue culture infective dose.

ruses. Antibodies directed against the HA surface glycoprotein are critical for protection; hemagglutination-inhibiting (HI) antibody titers of 1:40 or higher are considered protective (30, 80, 145). Previous experience with human influenza virus vaccines has shown that vaccines are effective only if the HA of the vaccine strain and the epidemic strain are antigenically closely matched (29, 31, 79, 93).

Avian Influenza Virus Vaccines

Several strategies are being explored to generate vaccines that will be effective in the event that a new pandemic influenza virus strain emerges in humans. These strategies draw on experience with human influenza vaccines. There are two licensed vaccines for human influenza in the United States: an inactivated virus vaccine and a live, attenuated vaccine. In this section we focus on the development of vaccines against avian influenza virus H9 (Table 3) and H5 (Table 4), subtypes because these are two of the subtypes that have infected humans.

The inactivated human influenza virus vaccine is an egg-derived inactivated trivalent vaccine containing 15 μg of each HA (H1, H3, and influenza B virus) that is administered by intramuscular injection. The components of the vaccine (currently two influenza virus A subtypes, H1N1 and H3N2, and an influenza B subtype) are determined each year by an international surveillance system coordinated by the WHO. The vaccine is in one of three forms: whole virus, which has been associated with adverse reactions in children and thus is little used; split-product vaccine, in which the virus has been detergent treated and highly purified; and surface antigen vaccine, which contains purified HA and NA (51). The influenza A vaccine strains are reassortant viruses that derive their HA and NA genes, and accompanying antigenic characteristics, from circulating influenza A viruses and their internal protein genes from influenza virus A/Puerto Rico/8/34 (PR8), which confer the property of high yield in eggs. The outbreaks of H1N1 influenza virus infection in 1976 and 1977 provided an opportunity for vaccine trials in naïve subjects (90, 133, 231). Differences in response to vaccination were observed between the younger, naïve population and the older, primed population. While a single dose of whole-virus vaccine, subunit vaccine, or split-product vaccine was sufficient to induce protective antibody responses in previously primed subjects, two doses of vaccine were necessary to achieve an acceptable antibody response in the immunologically naïve population. Whole-virus vaccines were significantly more immunogenic than subunit or split-product vaccines in naïve subjects, but the enhanced immunogenicity was associated with increased reactogenicity of the vaccine in children.

Inactivated H9N2 vaccines. Inactivated H9N2 influenza vaccines have been evaluated in animal models and humans and are presented in Table 3 (20, 76, 114, 159, 181). Three genetic lineages of H9 viruses have been defined, of which the prototype viruses are G1, G9, and Y439 (Korean) (67, 112). While these viruses have not caused lethal disease in humans, viruses from the G1- and G9-like lineages have been isolated from individuals with self-limiting respiratory disease and continue to circulate in poultry in China, remaining a potential source for further human infections (109, 137). A monovalent H9N2 whole-virus vaccine derived from A/HK/1073/99 (G1 like) administered intramuscularly, with or without aluminum adjuvant, was safe and well tolerated in clinical trials (76, 181). In a study reported by Stephenson et al., two doses of vaccine were required to induce HI antibody titers of ≥40 at any dose, but as little as 1.9 μg of HA was sufficient in the presence of the aluminum hydroxide adjuvant, while 15 μg was required in the absence of adjuvant (181). Interestingly, it was observed that half of the patients born before 1968 had baseline reactivity to H9N2 viruses, suggesting a preexisting or cross-reactive immunity (181). Two doses of vaccine were required to achieve HI titers of ≥40 in naïve individuals (<32 years of age), while one dose was sufficient in primed individuals (>32 years of age) (181). These observations are consistent with influenza vaccine studies performed during the 1976 to 1977 H1N1 outbreak (90, 133, 231). Based on these findings, one dose of an adjuvanted whole-virus vaccine may not be protective in an immunologically naïve population in the event of a pandemic and may leave a large proportion of the younger, naïve population unprotected. Development of an H9N2 vaccine containing a virus of the G9 lineage was undertaken based on the prevalence of the G9-like H9N2 influenza viruses in the bird population, coupled with its known transmission to humans, and the likely lack of sufficient cross-protection provided by a G1-like virus vaccine (20). To circumvent the poor growth of the G9-like H9N2 viruses in embryonated chicken eggs, a reassortant virus was generated with the high-growth PR8 virus, generating a virus expressing the HA and NA of the G9-like H9N2 avian virus and the internal proteins of the PR8 virus (20). Vaccination of mice with two doses of a vaccine prepared from the inactivated reassortant virus induced complete protection of the mice from infection with homologous and heterologous H9N2 influenza viruses (20). This vaccine is now being evaluated in a phase I clinical trial.

Inactivated H5N1 vaccines. A number of inactivated-vaccine candidates have been developed against H5 avian influenza viruses (Table 4). Due to the high pathogenicity of the H5N1 isolates targeted for vaccine development, approaches were designed in which propagation and work with wild-type virus would be minimized. Antigenically related surrogate nonpathogenic viruses and reassortant viruses were utilized for vaccine development. Lu et al. evaluated the efficacy of A/duck/Singapore/97, an H5N3 influenza virus isolate antigenically closely related to the 1997 H5N1 human virus isolates (92 to 93% amino acid sequence homology in HA_1 of HA) in mice (115). Two 10-μg doses of formalin-inactivated whole virus in the presence or absence of alum were required to elicit protective HI antibody responses (titer of ≥40) in mice against human H5N1 isolates; however, antibodies were cross-reactive for both identified antigenic groups of human H5N1 viruses isolated in Hong Kong in 1997. Furthermore, vaccination induced a high degree of protection from infection and death following challenge with a highly lethal human H5N1 virus. A phase I clinical trial of monovalent surface antigen A/duck/Singapore/97 vaccine, with or without MF59 adjuvant, was undertaken by Nicholson and colleagues (128). Volunteers received two doses (7.5, 15, or 30 μg HA) of vaccine 21 days apart by intramuscular injection. The vaccine was well tolerated, but NAb responses were poor in the vaccinees who received the nonadjuvanted vaccine. The most significant antibody responses occurred after two doses of 30 μg of vaccine,

TABLE 4. H5 subtype avian influenza virus vaccines

Vaccine type	Test system	No. of doses/route/vaccine strain/schedule	Notable outcomes (Ab/CMI/efficacy/other)	Reference(s)
Inactivated whole virus	BALB/c mice	2 doses/i.n. with 10 μg inactivated A/duck/Singapore/97 (H5N3) in absence and presence of alum/0 and 3 wk	2 doses ± alum required to elicit HI Ab titer in 65% of mice; detected Ab cross-reactive with H5N1 viruses/challenged 4 mo after second dose with HK/483 (H5N1), high degree of protection from infection and death, low viral titers in lungs, no dissemination to brain	115, 230a
	Humans	2–3 doses/i.m. with 7.5, 15, or 30 μg of inactivated A/duck/Singapore/97 ± MF59 adjuvant/0 and 20 days	Vaccine tolerated at all doses with no serious adverse effects/adjuvanted vaccine induced cross-reactive NAb to HPAI H5N1 human isolates/efficacy not determined	128, 179, 180
	BALB/c mice	3 doses/i.n. or s.c. with 20 μg formalin-inactivated H3N2 adjuvanted with *E. coli* LT/0, 1, and 2 wk	2 wk after final boost mice challenged with lethal heterologous HK/483 (H5N1), s.c. vaccinated mice completely protected from death, 50% of i.n.-vaccinated mice survived, protected against lethality and significant weight loss and had accelerated virus clearance from brains, nasal turbinates, and lungs	207a
	BALB/c mice	2 doses/i.m. with 10 μg formalin-inactivated H5N1/PR8 reassortant virus (modified H5-HK/491/97)/0 and 2 wk	Cross-reactive HI Abs produced/challenged 4 wk after second dose with 100 MID_{50}[a] A/HK/486/97, protected against lethal challenge, no evidence of viral replication in lungs/performed as well as formalin-inactivated wild-type virus	190
Inactivated subvirion	Humans	1–2 doses/i.m. with 7.5, 15, 45, or 90 μg of A/VN/1203/04/0 and 4 wk	Vaccine was well tolerated in healthy adults/at 90-μg dose 54% developed NAb titers and 58% HI Ab titers of 1:40 or greater; NAb titers of 1:40 or greater were seen in 43, 22, and 9% of subjects given two doses of 45, 15, and 7.5 μg, respectively	205
Subunit	Roosters	2 doses/10 μg HA and NA of A/HK/156/97 complexed with ISCOM/0 and 26 days	Strong Ab response only after 2 doses/challenged roosters 18 days after final vaccine dose with 2.5×10^3 $TCID_{50}$[b] of A/HK/156/97, all survived challenge without clinical disease/nonadjuvanted subunit vaccine did not protect against lethal challenge	152
	Humans	2 doses/i.m. with baculovirus-expressed recombinant H5 HA from A/HK/156/97/21, 28 or 42 days	No adverse events observed/frequency of NAb response was dose dependent, two doses of 25 or 45 μg required to elicit moderate increase in NAb titers to levels considered protective/efficacy not determined	206
DNA	BALB/c mice	2 doses/i.m. with 10 μg DNA encoding HA of A/HK/156/97/0 and 4 wk	4 wk after first vaccination no detectable Ab response, after boost 40% of mice developed low HI Ab titers/challenged 10 days after boost with 10 LD_{50}[c] CkHK97 or HK97, 100% protection against both homologous and heterologous virus challenge, 1/4 mice had moderate virus titers in lungs, no clinical signs of infection	100
	BALB/c mice	3 doses/i.m. with 100 μg DNA encoding NP and M of A/Puerto Rico/8/34/0 and 2 wk	Induced Ab against homologous virus proteins/activated T cells/challenged 1 wk after last dose with H5N1 viruses: HK/486 (low virulence), 17-fold-reduced viral replication in lungs; HK/156 (intermediate virulence), minor weight loss and ~500-fold reduction in lung viral titers; HK/483 (high virulence), 100% lethality	43
Live attenuated	Chickens	1 dose/i.v.,[d] IN, or i.t.[e] with $10^{7.8}$–$10^{8.7}$ EID_{50}[f] of recombinant virus A/HK/156/97 HA or A/HK/483/97 HA with all other genes from A/A nn Arbor/6/60 *ca*	Both vaccine strains were nonpathogenic for chickens/63–88% of vaccinated chickens developed HI Ab titers of 4–64 against homologous wild-type virus/challenged with homologous virus 20 days postvaccination, all chickens that seroconverted (HI Ab titer of >4) survived challenge/seroconversion rate was higher following i.v. vaccination than i.n. and i.t. vaccination	110

[a] MID_{50}, 50% mouse infectious dose.
[b] $TCID_{50}$, 50% tissue culture infective dose.
[c] LD_{50} 50% lethal dose.
[d] i.v., intravenously.
[e] i.t., intratracheally.
[f] EID_{50}, 50% egg infective dose.

and the highest seroconversion rates were seen when two doses of 7.5 μg of vaccine were administered with adjuvant. In a follow-up study reported by Stephenson et al., protective antibody responses were not detectable in any of the volunteers 16 months after the two doses; however, upon revaccination with a third dose, significant antibody responses were observed (179, 180). Sera from revaccinated volunteers were evaluated for cross-reactivity with human H5N1 isolates from 1997 and 2004, and they demonstrated an increase in seroconversion rates. These studies demonstrated that antigenically related surrogate nonpathogenic virus vaccines are weakly immunogenic but can induce cross-reactive antibodies when more than one dose is used and the vaccine is administered with an adjuvant.

Development of reverse genetic systems for the generation of recombinant influenza viruses has provided a safe and effective method for generating attenuated viruses with desired gene constellations entirely from plasmid DNA (46, 82, 127). High-growth recombinant viruses expressing a modified HA and unmodified NA from an H5N1 virus and the internal genes of PR8 were generated as potential vaccine candidates by using reverse genetics, followed by growth in embryonated chicken eggs (190). Site-directed mutagenesis was performed on the HA to change the basic amino acids adjacent to the cleavage site to amino acids found in nonpathogenic virus strains. The recombinant viruses were no longer lethal for chickens and mice but retained the antigenicity of H5N1 wild-type virus. Vaccination of mice with two doses of formalin-inactivated recombinant virus resulted in protective HI antibody titers (titer of 120) and provided complete protection from pulmonary virus replication and lethal virus challenge with homologous and heterologous H5N1 viruses. Vaccines generated based on this strategy using cell lines qualified for vaccines and using H5N1 viruses from 2004 are currently being evaluated in clinical trials (131, 217). A recent multicenter study evaluating an inactivated subvirion influenza virus H5N1 vaccine found that the vaccine candidate, while well tolerated in healthy adults, induced NAb or HI titers of 1:40 or greater in only 54 and 58% of the subjects, respectively, following two doses of 90 μg each (205). It is possible, however, that low levels of NAbs will be sufficient to provide protection in the event of an outbreak. Clinical trials assessing the immunogenicity of the vaccine in the elderly, children, and immunosuppressed individuals are currently in progress.

Live attenuated vaccines. FluMist, a trivalent cold-adapted (*ca*), live attenuated influenza vaccine was licensed for human use in 2003. The licensed *ca* live attenuated vaccine strains are generated by reassortment resulting in a virus with the surface glycoproteins of the target influenza virus strain and the six internal protein genes of the donor influenza virus A/Ann Arbor/6/60 *ca*. An attenuated *ca* virus was generated by passage of the H2N2 influenza A virus A/AnnArbor/6/60 at progressively lower temperatures, yielding a *ca* donor virus that exhibited the temperature-sensitive (*ts*), *ca*, and attenuation (*att*) phenotypes (117). Complete sequence analysis identified mutations in the coding regions of six of the viral genes, and the *att* phenotype has been shown to be the result of five mutations in three different gene segments (32, 91, 175, 188, 189). Monovalent, bivalent, and trivalent live attenuated vaccines for human influenza viruses have been tested extensively in humans for safety and efficacy (reviewed in reference 125). The presence of multiple attenuating mutations enhances the stability of the *ts* and *att* phenotypes and decreases the probability that reassortment between the attenuated vaccine strain and circulating influenza virus strains will result in a virus with enhanced virulence. Plasmid-based reverse genetic techniques have also been adapted for the generation of 6-2 reassortant live attenuated vaccines. Candidate live attenuated vaccines against H5N1 and H9N2 viruses were generated using reverse genetics (19, 110). Live attenuated avian influenza virus vaccines are being developed and evaluated in preclinical studies, and clinical trials are planned.

Subunit/expressed-protein vaccines. Rimmelzwaan et al. evaluated the efficacy of a subunit vaccine expressing the HA of A/HK/156/97, the index H5N1 avian influenza virus case, which is a highly virulent H5N1 virus strain (152). A nonadjuvanted vaccine formulation was compared to a formulation using the immune-stimulating complex (ISCOM) antigen presentation system, which consists of triterpenoid glycosides from the adjuvant QuilA and lipid in which the immunogen is incorporated. Chickens, which are highly susceptible to infection with H5N1 influenza virus, were vaccinated twice with 10 μg of each formulation 26 days apart and were then challenged 18 days after the last vaccination. Only the animals inoculated twice with the ISCOM preparation developed strong antibody responses and survived challenge without clinical signs of disease. These findings support the use of the ISCOM antigen presentation system, which can increase the efficacy of subunit vaccines. Treanor and colleagues evaluated the safety and immunogenicity of a recombinant baculovirus-expressed H5 HA vaccine derived from the same H5N1 virus in a phase I clinical trial in human volunteers (206). Volunteers were vaccinated intramuscularly with one or two doses of nonadjuvanted vaccine at a range of amounts (25 to 90 μg) at intervals of 21, 28, or 42 days. All doses of the recombinant H5 vaccine were well tolerated, but the vaccine was only modestly immunogenic at the highest dose. Twenty-three percent of volunteers had NAb titers of ≥1:80 after one dose of 90 μg, and 52% had NAb titers of ≥1:80 after two doses of 90 μg. Comparison of these results with those from earlier studies that evaluated antibody responses to conventional influenza vaccines in unprimed adult populations suggests that the recombinant H5 vaccine is suboptimally immunogenic (108, 147, 204).

DNA vaccines. Due to the risks of working with wild-type highly pathogenic H5N1 influenza viruses, DNA vaccine candidates targeting specific viral proteins have been evaluated. Kodihalli et al. immunized mice with DNA encoding the HA of A/HK/156/97 (H5N1) (100). Two doses of vaccine were required to induce HI antibody titers, and even then only 40% of the mice developed HI titers. However, vaccination protected mice from both homologous and heterologous virus challenge, and virus was not detected in the lungs or brains of the mice. Mice were also immunized with DNA encoding the HA from an antigenically related H5N8 influenza virus (100). Immunized mice were protected from death following challenge with A/HK/156/97; however, the mice showed transient signs of disease. Epstein and colleagues explored the efficacy of DNA vaccines expressing the conserved matrix (M) and nucleocapsid (NP) proteins of PR8, an H1N1 influenza virus, against H5N1 isolates (43). Vaccination of BALB/c mice with 100 μg

of each plasmid three times at 2-week intervals protected mice from challenge with a low-virulence H5N1 virus, decreasing viral lung titers by 17-fold. Although mice challenged with an H5N1 virus of intermediate virulence also had reduced pulmonary viral titers, weight loss was observed, and vaccination provided no protection against challenge with a highly lethal H5N1 influenza virus. In an avian influenza pandemic, a DNA vaccine encoding an HA antigenically related to the pandemic strain, or other viral proteins, may offer protection from severe disease and death until a genetically matched HA vaccine can be made.

The development of avian influenza virus vaccines has taken on a degree of urgency, with the spread of H5N1 avian influenza virus in birds through a large part of the world and the growing number of human infections in Asia and Europe. Past experience with influenza virus vaccines supports the development of whole-virus (inactivated or live attenuated) vaccines. While DNA vaccines show promise in animal models, their efficacy in humans has not been well established. The generation of vaccines by using reverse genetic techniques greatly reduces the dangers inherent with working with highly pathogenic viruses.

VACCINES: FROM CONCEPT TO CLINICAL USE

The Food and Drug Administration's (FDA) Center for Biologics Evaluation and Research (CBER) is responsible for regulating vaccines in the United States. While recent advances in virology often lead to more rapid identification of pathogens and implementation of strategies to target specific pathogens, moving a vaccine candidate from research to licensure still takes on average more than 10 years (184). The specific issues related to vaccine development for different pathogens make it difficult to estimate the time required from concept to licensure. Time is an important factor in the development of vaccines against emerging pathogens, especially those with pandemic potential. The research and development of vaccines is an expensive and complex process that includes both preclinical and clinical testing phases evaluating the safety and efficacy of the proposed vaccine formulation. Here we briefly describe the stages of development through which all vaccine candidates must progress before licensure. For a thorough review of the stages of vaccine licensure, see reference 166, and for a review of the development of vaccines against potential pandemic strains of influenza see reference 116.

Preclinical Studies

Before clinical trials assessing vaccine candidates can be carried out, investigators are required to submit an Investigational New Drug (IND) application to the FDA. The IND application describes the method of vaccine manufacture and provides data regarding the safety of the vaccine formulation, including systemic toxicology, sterility, cell line characterization, and endotoxin levels. A battery of tests must be performed to demonstrate that adventitious agents have not been introduced into the vaccine formulation during the manufacturing process. The IND application must also contain data from studies evaluating the vaccine strain itself, as well as the immunogenicity and efficacy of the vaccine candidate in animal models. The currently licensed live attenuated influenza virus vaccines are produced in specific-pathogen-free embryonated chicken eggs. Licensure of selected cell lines for the growth of vaccine candidates is currently being evaluated. The use of cell lines in place of embryonated eggs for vaccine candidate growth would allow manufacturers to increase both the scale and rate of production and would alleviate dependence on the availability of approved embryonated eggs.

In vitro studies. Studies performed prior to introduction of live attenuated avian influenza virus vaccine candidates into humans include complete sequence analysis to ensure that the desired viral genotypes and gene constellations have been maintained, without the introduction of unwanted mutations during the manufacturing process.

In vivo testing in animal models. Prior to initiating clinical trials, the immunogenicity and efficacy of vaccine candidates must be evaluated in appropriate animal models. The necessity to provide data from studies in animal models is an obstacle for vaccine development for some pathogens. As described for SARS-CoV, animal models that exactly mimic the viral pathogenesis and disease progression observed in humans have not been identified; however, the currently available animal models allow for the evaluation of vaccine candidates by measuring the efficacy of preventing or reducing viral replication, inducing an immune response, and protecting from histopathologic evidence of disease in some models. A further limitation encountered for research with both SARS-CoV and avian influenza viruses is access to animal facilities with the required level of biological containment. Avian influenza virus vaccine candidates can be evaluated in mice or ferrets if these species support replication of avian influenza viruses. The immunogenicity of vaccine candidates is determined by evaluation of NAbs and HI antibodies induced by vaccination in these animal models. By convention, for most viruses a fourfold increase in NAb titers following vaccination is strongly indicative of a protective immune response. Efficacy of the vaccine candidates is also evaluated in animal models by challenge of vaccinated animals with homologous and heterologous wild-type viruses. These data provide evidence for protection against the wild-type target virus strain and for cross-protection that may be provided by vaccination. Influenza viruses can undergo antigenic drift relatively rapidly, and with the uncertainty as to which virus may have the greatest pandemic potential, vaccine candidates that provide some degree of protection against viral variants would be preferable.

Once the preclinical data have been evaluated and determined to be acceptable by the FDA, vaccine candidates can be evaluated in clinical trials.

Clinical Trials

Clinical trials are carried out in three phases. Phase I trials, which generally involve tens of subjects, evaluate the safety and immunogenicity of vaccine candidates in human volunteers and provide guidance as to dosing in future studies. Phase II trials evaluate safety, immunogenicity, and dose ranges and generally involve hundreds of subjects, while phase III trials evaluate the safety, immunogenicity, and efficacy of vaccine candidates and usually involve a large number of test subjects. Clinical studies involving vaccines targeting highly pathogenic

respiratory pathogens can present regulatory and strategic obstacles. In the case of live attenuated avian influenza virus vaccines, studies will be carried out at in-patient facilities at a time when human influenza viruses are not circulating widely, and viral shedding will be closely monitored.

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