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STREAM 3
Background document

MINIMIZING IMPACT

Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza

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1. Disease Burden and Epidemiology

1.1. Seasonal influenza

Influenza is a highly contagious respiratory disease thought to spread by droplet, contact and airborne routes. Respiratory droplets are currently thought to be the main mode of transmission. There is also some suggestion that influenza may spread through airborne aerosols that are expelled through coughing or sneezing or aerosol generating procedures such as bronchoscopy or intubation. The disease, characteristically a febrile illness with respiratory symptoms, ranges in severity from mild to debilitating and can lead to lethal primary fulminant pneumonia, particularly in persons with underlying pulmonary or cardiopulmonary pathologies. Adults are most infectious from 1 day before symptom onset up to 5 and even 7 days afterwards. Rates of infections are highest among young children who shed virus longer and are a potential source of infection for older age cohorts [1]. Rates of serious illness, complications and death are highest in persons aged 65 years and older, and in persons with chronic cardiac or respiratory conditions. Hospitalizations are common in young children. The great majority of influenza-related deaths can be attributed to secondary bacterial pneumonia caused by common respiratory pathogens such as pneumococci, group A streptococci and staphylococci [2].

Epidemics and outbreaks of influenza occur in different seasonal patterns, depending on location: in temperate climate zones, seasonal epidemics typically begin in autumn and peak in mid-winter, infecting about 5-15% of the population each season; in tropical zones, seasonal patterns are less pronounced; and in some regions, the virus can be isolated year-round but there are periods of increased transmission [3]. Many research questions about the disease in tropical regions still remain unanswered [3a].

The repetitive occurrence of yearly influenza epidemics is maintained through the ongoing process of "*antigenic drift*", driven by the accumulation of mutations in the two viral surface proteins, hemagglutinin (HA) and neuraminidase (NA), leading to the constant emergence of new virus variants. Antibody pressure likely selects for the new variants, against which there is little or no pre-existing immunity in the population. For this reason, seasonal epidemics of influenza occur each year and the virus strains to be included in that year's vaccine must be chosen to match the emerging new variants [4].

The burden of influenza is difficult to determine accurately. Current estimates in the USA call for 25-50 million cases of illness (up to 20% of the population), leading to 150 000-200 000 hospitalizations and 30 000-40 000 deaths per year [5]. If one extrapolates these figures worldwide, the average global burden of seasonal influenza comes to be on the order of 600 million cases, 3 million cases of severe illness and 250 000-500 000 deaths per year. A significant disease burden has been recognized in young children, which led US public health officials to recommend vaccination of all healthy children, initially aged 6-23 months, now up to 18 years of age [6]. Hospitalization rates from severe illness can be as high as 3 per 1000 for 6 to 23 month-old children and 9 per 1000 for children younger than 6 months [7].

The average seasonal incidence of influenza-related deaths in the USA from 1972-1992 was calculated to be 9.1 per 100 000 persons, about 1% of all deaths in the country [2]. Yearly seasonal influenza-associated excess mortality in the decade 1990-2000 was estimated to be 14, 16, 19.6 and 21.6 per 100 000 population in the Netherlands, Germany, the USA and Switzerland, respectively [8]. In the USA, rates of visits to clinics and emergency departments attributable to

influenza were highest among children 6 to 23 months of age, reaching 56 visits per 1 000 children in the 2002-2003 influenza season and 122 per 1 000 in the 2003-2004 season [9]. At particular risk for infection are infants with several older siblings, with the highest rates of serious influenza-related illness occurring in 6-12 month-old infants [10]. The yearly burden of influenza among people over 75 years of age in France from 1980-1990 was estimated to be 216 hospitalizations per 100 000 persons, similar to the estimated rate of 160 per 100 000 persons in the USA in the same age group [2,11].

The evidence of a disease burden associated with non-pandemic influenza in the tropics has been documented by recent studies on hospitalizations and deaths in Hong Kong [12] and in rare investigations of influenza outbreaks in poor developing countries. Little attention has been paid, however, to influenza among infants in these countries [13]. A recent study showed a high burden of influenza among under one-year-old children in Hong Kong, with hospitalization rates varying from 3.9 to 10.4 per 1 000 children per year [14]. A recent study in Thailand showed that the influenza virus was a leading cause of pneumonias, hospital admissions and febrile illnesses leading to outpatient clinic visits during 2003-2004, with an estimated 1420 cases per 100 000 outpatients [15]. Here also, young children and the elderly were the most affected groups.

Finally, it must be emphasized that the primary economic impact of seasonal influenza is from lost wages, as the disease may cause 10-12% of all absences from work [16,17]. Influenza vaccination has been shown to reduce absenteeism and health care costs in working adults [18].

1.2. Pandemic influenza

Due to the segmented nature of the influenza virus genome, circulating human influenza virus A strains can acquire new genes from an avian or other animal influenza virus. This process is believed to occur most readily in pigs, as pigs have the complete set of sialylated receptors for avian, swine and human influenza virus strains [19,20]. Co-infection in pigs can thus result in the emergence of a virus with a completely new subtype, referred to as an "*antigenic shift*". If the new virus can efficiently spread to humans, a worldwide pandemic can occur, as was the case in 1957 and 1968. In other instances, an avian or swine virus can cross the species barrier, become adapted to humans and initiate a pandemic, as was the case in 1918 and is the case with the pandemic (H1N1) 2009 virus [21,22,22a].

Before the 2009 pandemic, the impact of a new influenza pandemic had been grossly estimated at 1-2 billion cases of influenza, 5-12 million cases of severe illness, and 1.5-3.5 million deaths worldwide [23]. Historians estimate that more than 50 million people died in the 1918-1920 influenza pandemic [24]. In the USA, the impact of a new pandemic, assuming it would be of similar magnitude to the 1957 or 1968 pandemics, is projected to be 18–42 million outpatient visits, 314 000–734 000 hospitalizations and 89 000–207 000 deaths.

Avian A (H5N1) panzootic

The threat of a new influenza pandemic became a global concern in recent years after the emergence of the H5N1 avian influenza virus in 1997 in Hong Kong, resulting in the death of 6 of the 18 affected patients. Fortunately, the virus was not able to spread from person-to-person and the outbreak was controlled through massive culling of poultry. H5N1, however, reemerged in 2003–2004 in China, Japan, South Korea and South East Asian countries. More than 60% of human patients diagnosed with the virus died. In May 2005, a variant of the highly pathogenic

(HP), Asian lineage, H5N1 emerged in wild birds in Qinghai Lake, China, that not only killed domestic poultry but also wild aquatic birds and mammals [25,26]. Increased virulence of this HP strain has been linked to mutations in the polymerase genes PB1 and PB2, and in the NS1 gene [27]. The HP variant subsequently spread to several countries in Asia, Africa and Europe, where it has repeatedly been recovered from migratory birds and been the cause of outbreaks in poultry. The virus now appears enzootic in several countries, including Nigeria, Egypt, Bangladesh, Vietnam and Indonesia, while showing continuous sequence evolution leading to the emergence of different molecular clades and sub-clades [28].

Cases of human H5N1 infections have been reported in many countries, but there have been only a few instances of well-documented human-to-human transmission among close contacts. Although the H5N1 virus continues to be a zoonotic virus, and human infections remain rare, it has been considered prudent to prepare for the 'worst case' scenario, given the alarming 60% average case fatality rate of H5N1 influenza in humans [29].

Other avian influenza viruses have occasionally caused human infections, such as H9N2 in 1999 in Hong Kong, an H7N7 outbreak in 2003 in the Netherlands, which caused 89 confirmed human cases with conjunctivitis and one death, and H7N2 and H7N3 infections in 2003–2004 in North America.

Pandemic (H1N1) 2009 influenza

The emergence of the pandemic (H1N1) 2009 influenza virus in humans in early April 2009 in Mexico and California came as a total surprise. The H1N1 strain quickly spread worldwide through human-to-human transmission, generating the first influenza pandemic of the twenty-first century. Most cases are diagnosed clinically and are not laboratory-confirmed [30]. Thus, the actual number of people who have been infected by the pandemic (H1N1) 2009 influenza virus is not known. The rates of H1N1 respiratory illness in the northern hemisphere have been increasing with the approaching 2009/10 winter. Some estimates call for more than 1 billion people to be infected during the coming months.

The H1N1 virus is antigenically distant to human seasonal influenza viruses but genetically related to three viruses that circulate in pigs [30a]: a triple reassortant H3N2 swine virus with genes from classical swine H1N1 mixed with genes from North American avian and human H3N2 influenza viruses [20,31,32], an 'avian-like' swine H1N1 virus which was first isolated from pigs in Belgium in 1979 and which gradually replaced classical swine H1N1 viruses throughout Europe and Asia [33,34], and the classical swine H1N1 virus. The H1 sequence can actually be traced back to the 1918 H1N1 pandemic virus (the "Spanish flu"), which has remained endemic in swine and continued to circulate among pigs in Asia and the Americas [35,36].

The pandemic (H1N1) 2009 influenza virus, therefore, has virus gene segments that are swine, human and avian in origin: its HA, NP and NS gene segments come from the classical swine virus, its NA and M segment from the avian-like Eurasian reassortant lineage, and its PA and PB2 segments from the North American avian lineage. The reassortment of these different lineages probably occurred years before the virus emerged in humans [37-39]. Surprisingly, there has been no evidence that pigs have any role in the epidemiology or in the worldwide spread of the virus in human populations [40].

A characteristic feature of pandemic (H1N1) 2009 is that it mostly involves children and young adults. An early US study showed that 60% of patients were 18 years of age or younger [41]. The majority of H1N1 cases have occurred in young people, with a median age estimate of 12 to 17 years, whether in Canada, the USA, Chile, Japan or the UK. This speaks in favor of some immunity to the virus in the older population; subsequent studies have shown that 33% of individuals over 60 years of age had cross-reacting antibodies to the pandemic (H1N1) 2009 virus by hemagglutination inhibition and neutralization tests [37,42]. Although no neutralizing antibodies against the pandemic (H1N1) virus could be found in another study in sera from people born after 1920 [43], limited cross-reactive antibodies to the pandemic (H1N1) 2009 virus were detected in serum samples from older adults in trials of a seasonal trivalent inactivated vaccine predating the current pandemic [44]. The possible role of the NA antigen in cross-protective immunity [44a] should not be overlooked. In addition, more than 50% of the T cell epitopes (whether T-helper or CTL epitopes) in the pandemic (H1N1) 2009 virus seem to be shared with the seasonal influenza virus strains used to prepare the 2008 influenza vaccines [45].

In most cases, pandemic (H1N1) 2009 is a mild, self-limiting upper respiratory tract illness with fever, cough and sore throat. Some patients have experienced gastrointestinal symptoms including diarrhea and vomiting. The spectrum of clinical presentation can actually vary from asymptomatic to primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multi-organ failure and death. Of note, 2% to 5% of confirmed cases in the USA and Canada and 6% of cases in Mexico had to be hospitalized, a fifth of them requiring management in an intensive care unit. The rate of hospitalization could be as high as 10% in some cities. Most, but not all, of the hospitalized patients had underlying medical conditions such as cardiovascular disease, respiratory disease (including asthma), auto-immune disorders, obesity, diabetes or cancer. Pregnant women, especially in their second and third trimester, also were found to be at increased risk for severe disease [46].

On the basis of recorded clusters in the USA, the household secondary attack rate for pandemic (H1N1) 2009 was estimated to be 27.3%. In school outbreaks, a typical schoolchild infected, on average, 2.4 (range 1.8 to 3.2) other children within the school. The basic reproductive number, R_0, thus has ranged from 1.3 to 1.7. This is consistent with further pandemic spread causing illness in 25% to 39% of the world's population over a 1-year period, similar to the spread of the 1957-1958 Asian influenza A (H3N2) pandemic [46a].

The overall (H1N1) case fatality rate in Mexico was estimated to be about 0.4% [47]. Preliminary estimates of the overall case fatality ratio are <0.5%. Population-based age-specific mortality rates are highest in 50 to 60 year old persons. In contrast, fatal disease occurs most often in the >65 year-old age group during seasonal epidemics. In a recent study of the first 16 weeks of the pandemic (H1N1) 2009 in California which saw 1088 cases of hospitalization or death, the median age of hospitalized patients was 27 years of age. The overall fatality rate was 11% and was higher in persons 50 years of age and older [47a].
Human-to-human transmission of the pandemic (H1N1) 2009 virus appears to be similar to transmission of other human influenza viruses (e.g., seasonal influenza) occurring primarily either directly or indirectly through close unprotected contact with large respiratory droplets. The contribution of close range exposure to smaller droplet nuclei to transmission of influenza is unknown, but may be more prominent under special conditions e.g., aerosol-generating procedures associated with increased risk of infection transmission. Influenza is also likely transmitted through contact with fomites contaminated with respiratory or possibly gastrointestinal fluids [48].

2. Influenza Vaccines

2.1. Overview

Currently, available seasonal influenza vaccines are made from either inactivated (detergent-split or whole virion) or live attenuated influenza virus (LAIV), usually propagated in the allantoic cavity of embryonated chicken eggs from certified farms [49]. However, some vaccines are now produced using mammalian cell lines, such as MDCK, PERC-6 or Vero cells [50]. Seasonal vaccines include two currently circulating influenza A virus strains and one influenza B virus strain. These trivalent vaccines have been used for decades in industrialized countries to prevent seasonal influenza infection. They provide a high benefit/cost ratio in terms of preventing hospitalizations and deaths, as shown in numerous studies on vaccination of the elderly and of individuals at high risk for severe outcomes of influenza [51-53].

WHO estimates that there are globally about 1.2 billion people at high risk for severe influenza outcomes: 385 million elderly over 65 years of age, 140 million infants, 75 million pregnant women, and 700 million children and adults with an underlying chronic health problem. In addition, 24 million health-care workers should be immunized to prevent spreading of the disease to high-risk populations. The world's total vaccine production capacity at this time, is about 850 million doses of trivalent vaccine [56].

No seasonal influenza vaccine can offer protection against an emerging pandemic influenza virus strain, whether of avian (H5N1, H7N7 or H7N2), equine, or swine (pandemic (H1N1) 2009) origin; specific 'pandemic vaccines' need to be prepared for that purpose. Given the world's current vaccine production capacity, if a monovalent inactivated pandemic influenza vaccine was to be produced according to the formulation of seasonal vaccines (i.e. 15 µg HA per dose), as recommended for the current (H1N1) 2009 vaccine, only about 875 million people could be vaccinated [54]. In addition, the manufacturing process for a conventional influenza vaccine may take up to 6 months, an unacceptable delay in the event of a sudden pandemic.

The development of pandemic influenza vaccines also raises complex challenges, such as ensuring that sufficient seasonal influenza vaccine will still be available, estimating with accuracy short- and medium-term production capacity of the different producers and reserving part of the foreseen production capacity for poor countries with no or little access to the vaccine [55,56].

Hence, the importance of developing influenza vaccines with broader immunogenicity, of finding well tolerated adjuvants with an antigen dose-sparing effect [57] or, if possible, of designing 'universal' influenza vaccines that could circumvent the need for yearly production of 'seasonal' vaccines.

2.2. Inactivated vaccines

2.2.1. Split influenza vaccines

- Seasonal split inactivated vaccines

Most seasonal influenza vaccines produced today are 'split-vaccines' (subvirion vaccines) which result from detergent-treatment of formalin- or betapropiolactone-inactivated purified influenza virus. Trivalent split inactivated influenza vaccines contain 15 µg HA from each of three

circulating viral strains. These vaccines have a remarkable safety profile, including in 6 to 23 month-old children [58,59].

A rare, severe adverse event, neurologic Guillain-Barré syndrome (GBS), was associated with influenza immunization with an inactivated vaccine in 1976 when ~45 million people in the USA were vaccinated against an A (H1N1) S-OIV that had initially infected soldiers at Fort Dix, NJ. The vaccine-attributable risk was 8.8 GBS cases per million vaccinees in the 6 weeks after vaccination, as compared to 0.7 to 4.6 cases per million persons in the unvaccinated population [60]. The origin of this adverse event has never been fully elucidated.

Multiple studies have shown inactivated influenza vaccines to be approximately 60% to 90% efficacious against influenza illness in healthy children and adults, depending on the antigenic match between the circulating and vaccine viral strains [61-64]. In vaccine-naive children less than 10 years old, a two dose schedule of inactivated vaccine is required [65]. In contrast, a second dose of vaccine in elderly individuals does not boost immunity [66].

- Pandemic split inactivated vaccines

Immunogenicity trials with pandemic split inactivated H5N1 candidate vaccines showed disappointingly low immune potency, as two 90 µg haemagglutinin doses of split vaccine at four weeks interval elicited responses in only 35% of elderly volunteers [67-69]. Addition of aluminium salts as an adjuvant had a limited effect on the potency of the vaccines [70]. In contrast, remarkable results were obtained with adjuvants such as polyoxidonium (Microgen), MF59 (Novartis), AS03 (GSK) or AF03 (Sanofi Pasteur), which are based on squalene-derived oil-in-water emulsions, and which showed remarkable 'dose-sparing' properties, allowing a reduction in the amount of hemagglutinin in the vaccine to $\leq$ 7.5 µg per dose [71-74]. In addition, the adjuvanted H5N1 vaccines elicited broadly cross-neutralizing antibodies that protected ferrets from challenge with virus strains from different H5N1 virus clades [75]. The adjuvants were, however, associated with increased rates of local reactogenicity.

It has been suggested that the addition of adjuvants to seasonal influenza vaccines might be of benefit to populations at risk, such as persons with underlying chronic diseases [76]. One of the Novartis seasonal influenza vaccines is actually adjuvanted with MF59.

In the case of pandemic (H1N1) 2009, and despite lack of immunity to the virus in the human population, a one-dose schedule of immunization with a 15 µg HA-containing, unadjuvanted, inactivated split influenza vaccine elicited protective immune responses in the adult population [77,78]. Thus, among healthy US volunteers who received a single 15-µg dose of either the Sanofi-Pasteur or the CSL Limited inactivated split (H1N1) 2009 vaccine, a robust immune response was documented in 96% and 80% of 18-64 year-old, respectively, and in 56% and 60% of 65 year-old and older adults, respectively [79]. In children less than 10 years, however, a two-dose regimen will be required. The robust immune response to the pandemic (H1N1) 2009 vaccine observed in the 18-49 year-old volunteers was unanticipated and suggests that there may be more similarity between the pandemic (H1N1) 2009 influenza virus and recent seasonal influenza virus strains than has been recognized so far.

The use of adjuvanted pandemic vaccines has allowed the amount of HA antigen to be decreased in the current pandemic H1N1 vaccine. However, it may raise regulatory problems in the USA, where an adjuvanted influenza vaccine has never been licensed, contrary to Europe [80].

Complete safety and immunogenicity data on the pandemic (H1N1) 2009 influenza vaccines in adults are unlikely to be available before the end of December 2009 and will not be available before February 2010 for children.

2.2.2. Whole-virion influenza vaccines

Inactivated whole-virion vaccines were found to have better immunogenicity in naive individuals than split vaccines, but may be associated with febrile reactions, particularly in children [81]. H5N1 inactivated whole-virion vaccines have been produced by several companies. Adjuvantation with aluminium hydroxide was found to be devoid of an effect on the immunogenicity of the Vero cell-derived Baxter H5N1 vaccine [82], whereas it provided high immunogenicity to the H5N1 Nobilon and Omninvest vaccines. The latter required only a single IM dose of 6µg HA [83], whereas both the Sinovac vaccine [81] and the Baxter vaccine [82] necessitated two doses of vaccine with 7.5 µg HA. This topic has been extensively reviewed [84].

2.3. Live Attenuated Influenza Vaccines (LAIV)

The second major approach to influenza vaccines has been the development of live attenuated influenza vaccines (LAIV) that can be administered by the intranasal route in the form of sprays and multiply in the upper respiratory tract mucosa [84a].

2.3.1. Cold-adapted (ca) influenza virus strains

Growth of influenza viruses at mild temperatures led to the selection of cold-adapted (*ca*) virus strains, which grow well in primary chicken kidney cells and embryonated eggs at 25–33°C, have a reduced replication rate at 37°C, and show attenuated virulence in ferrets. Seasonal LAIVs are obtained by reassorting the HA and NA genomic segments from a circulating influenza virus strain with the other six genomic segments from a recipient strain that carries the *ca* mutations. Despite exhaustive testing, reversion of LAIV to wild-type phenotype has never been documented [85].

A trivalent *ca* LAIV developed by Microgen has been in use for decades in Russia to immunize millions of people every year. A trivalent *ca* LAIV (Flumist^{TM}) has been developed for intra-nasal spray delivery by MedImmune and Wyeth in the USA, where it is licensed for individuals 2-59 years-old. The vaccine was proven highly efficacious in Phase III trials, showing a 92% overall protection rate over a 2-year study in children [86-88]. Other LAIVs are in development using *ca* virus strains grown in Vero cells or in Madin-Darby Canine Kidney (MDCK) cells on microcarriers at 25°C.

LAIV was found to be well tolerated and effective in children as young as 6 months of age [89] and to provide herd immunity to adults when used in children [90]. It also demonstrated superior efficacy compared to the trivalent inactivated vaccine in preventing influenza illness in young children [91], even those with recurrent respiratory tract infections [92] or asthma [93].However, in healthy adults followed up during the 2007-2008 season, LAIV was less efficacious than the inactivated vaccine in the prevention of laboratory-confirmed, predominantly H3N2, symptomatic influenza A (36% versus 68%) [94].

Nasal shedding of LAIV in individuals 5-49 years of age was found to generally be of short duration (1 to 10 days post immunization) and at low titers [95]. Transmission of the attenuated virus to contacts has been documented only once in a single person who remained asymptomatic.

The role of cell-mediated immunity in LAIV-elicited protection against influenza illness was documented in a large efficacy trial in 6 to 36 month-old children in the Philippines and Thailand, showing strong correlation of the cellular immune response with protection [96].

LAIVs are attractive pandemic vaccines because they are cheaper and quicker to manufacture than traditional inactivated vaccines. A series of LAIVs were produced by MedImmune (USA) by reassortment between avian influenza A viruses H5N1, H7N3 or H9N2 and the *ca* Ann Arbor virus strain (H1N1). Phase I clinical trials of resulting *ca* reassortants were performed on volunteers kept in an isolation unit at Johns Hopkins Medical Center, Baltimore, MD, USA. Virus shedding into nasal secretions was detected for one day in 60% of volunteers and for up to four days in another 25%. After a second immunization a few weeks later, no viral shedding could be detected. A two-dose immunization with the H5N1 LAIV fully protected mice and ferrets against pulmonary replication of homologous and heterologous wild-type H5N1 virus strains [97]. In the (H1N1) 2009 outbreak, the H1N1 LAIV vaccine was the first to be administered in the USA. The US government placed an order for nearly 40,000,000 doses to be delivered first to target groups and later to the remaining US population that desired it.

A H5N2 avian-human *ca* reassortant influenza virus strain was developed as a LAIV by Microgen, Russia, [98] and successfully tested in Phase I and Phase II clinical trials using two immunizations 21 days apart.

2.3.2. Other live attenuated influenza vaccines

A different type of LAIV has been developed by Green Hills Biotech, Vienna, Austria, by deleting the NS1 viral gene, which encodes a virulence factor known to antagonize interferon, from a H5N1 avian strain. The resulting ΔNS1 H5N1 strain was grown in Vero cells, showed attenuation in mice and ferrets, and provided protection of the animals against virulent challenge with wild-type H5N1 virus strains [99].

2.4. Other types of Influenza vaccines

2.4.1. M2e-based vaccines

M2 is an integral membrane protein of influenza virus: it forms tetrameric proton channels in the membranes of virus-infected cells [100]. Its extracellular domain, M2e, a 23 amino acid-long peptide, is remarkably conserved between H1N1, H2N2 and H3N2 influenza A virus strains [101] and could serve as an 'universal' influenza A vaccine [102]. The M2 protein is scarcely present on the influenza virion, but is abundantly expressed on virus-infected cells. Passive administration of anti-M2e antibodies affords significant protection against influenza A virus challenge in animal models. The mechanism of protection is believed to be NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), not virus neutralization.

A recombinant particulate vaccine was engineered by genetically fusing copies of the M2e domain to the hepatitis B core antigen (HBc). The resulting (M2)-HBc fusion protein spontaneously assembled into highly immunogenic virus-like particles (VLPs) that provided

complete protection against a lethal influenza A virus challenge in mice and ferrets [103]. The product was developed into a vaccine by Acambis, Cambridge, MA, USA.

A M2e vaccine formulation was also developed as a fusion protein between the CTA1-DD adjuvant and tandem repeats of the M2e sequence [104,105]. A subunit vaccine made of the M2e peptide covalently coupled to the well-conserved NP internal protein, a known target for cytotoxic T cells, and to the ISS adjuvant [106], is in development at Dynavax Technologies, Berkeley, CA, USA.

Another approach has been developed at VaxInnate, USA, linking four tandem copies of M2e to *Salmonella typhimurium* flagellin type 2 protein. The resulting STF2.4xM2e fusion protein, which can be produced in high yields in *E coli* fermentors, was shown to induce a robust and long-lasting protective antibody response in mice [107].

2.4.2. Subunit vaccines

Attempts were made at developing a pandemic influenza subunit vaccine by using purified HA protein from influenza virus H5N1 expressed in a baculovirus expression system. The vaccine was well tolerated but showed poor immunogenicity in human volunteers, probably due to lack of oligomerization of the antigen [108,109]. A seasonal trivalent vaccine produced in the baculovirus system (Protein Sciences Corp.) has been clinically evaluated in phase 3 studies with some success andhas been submitted to regulatory authorities for evaluation.

Attempts at producing recombinant HA are also being made using transgenic plants or filamentous fungi.

Crucell is commercializing an influenza vaccine based on virosomes, with the HA and NA surface spikes of the three currently circulating influenza virus strains inserted into the membrane of liposomes. A nasal formulation of the vaccine had to be withdrawn from the market, due to undesirable neurological side effects (Bell's palsy) linked to the *E. coli* labile toxin (LT) used as an adjuvant. Other formulations of influenza vaccines for mucosal delivery are in progress including immunostimulating complexes (ISCOMs).

2.4.3. Virus-like particles (VLP)

Influenza VLP have been produced in insect cells using recombinant baculovirus vectors that express the viral proteins HA, NA and M1 [110,111]. The VLP induced both humoral and cellular immune responses in mice [112]. H5N1 VLP developed by Novavax, Rockville, MD, USA, elicited strong hemagglutination-inhibiting (HAI) antibodies and cell-mediated immune responses in mice and ferrets, and protected 100% of the animals against cross-clade H5N1 virus challenge at the low dose of 6µg HA [113,114]. A two-dose Phase I trial is planned shortly.

2.4.4. Live recombinant vaccines

Live recombinant vaccines have been engineered using either non-replicative adenovirus Ad5 [115] or modified vaccinia Ankara (MVA) virus 116] as vectors. These vaccines are still at a preclinical development stage [117,118]. A MVA recombinant which will express all five, M1, M2, HA, NA and NP, proteins is being developed at this time.

2.4.5. DNA vaccines

Influenza DNA vaccines, which would have many advantages, including ease of manufacture and storage, showed remarkable efficacy in mouse models but their development has been hampered by the limited immunogenicity of naked DNA in humans, especially in terms of induction of humoral immunity [119].

2.5. Correlates of protection

Correlates of vaccine protection have been extensively reviewed [120,121]; it is generally accepted that:

HA-specific, neutralizing antibodies are responsible for protective immunity against infection by closely matched influenza virus strains; they are readily elicited by inactivated influenza vaccines and LAIVs and serve as immune correlates of protection. They can be measured by either microneutralization tests or hemagglutination-inhibition tests.

NA-specific antibodies limit virus spread and are responsible for protection against severe disease and mortality. Immunity to NA is probably partially cross-protective, at least within virus sub-types [44a].

M2e-specific antibodies could be involved in cross-protective immunity relying on antibody-dependent cytotoxicity (ADCC). Such an immunity would extend to most human influenza A viruses.

NP contains T cell epitopes that elicit NP-specific cytotoxic T cells (CTLs) that can lyse influenza virus-infected cells in a cross-subtype fashion. This cellular immunity should provide cross-subtype protection against severe disease and mortality.

3. Issues to be addressed

Immunization against influenza is an essential public health intervention to control both seasonal influenza epidemics and pandemic influenza. However, many countries, particularly those that are under-resourced, have not developed policies to vaccinate their people at risk for seasonal or pandemic influenza.

This is related, in part, to insufficient local information on the burden of influenza disease, its seasonality, and its social, economic and health impact. Influenza disease burden data are not available for most low-income developing countries. Collecting information on yearly influenza tolls in these countries will require well-designed incidence and prevalence studies, such as those that are being carried out in Thailand, Senegal and a few other countries. These will potentially be a powerful drive for the development of appropriate public health policies and will encourage seasonal vaccination in these countries at dates of the year that remain to be determined.

There also are marked differences between countries in terms of potential access to influenza vaccines, resources to produce and distribute the vaccine, and capacity to establish seasonal influenza vaccination programs. During a pandemic, these differences are further accentuated as limited quantities of vaccine can be made available worldwide. Equitable access to a pandemic vaccine is a critical issue to be discussed. There are several developing countries which plan to

produce seasonal influenza vaccines and have the necessary vaccine production facilities. It is important to encourage the development of further production facilities in developing countries and to help transfer procedures, expertise and intellectual property to local manufacturers, as WHO has started by establishing a technology transfer platform at the Netherlands Vaccine Institute. In the context of a new pandemic, another critical issue will be the distribution of the vaccine and prioritization of risk-groups.

Seasonal vaccines present significant challenges as they must be ready on time, clinically evaluated for safety and immunogenicity and updated every year. Improvements of vaccine formulations that could provide broader range of protection against antigenically drifted virus strains would reduce the frequency of vaccination and provide much better protection against newly appearing seasonal virus strains. The design of influenza vaccines eliciting broad protection against a variety of virus subtypes remains a high priority objective for research. Furthermore, eliminating the need for annual vaccination would help in making vaccination programs easier to establish. Another bottleneck to the production of seasonal vaccines is the limited availability of embryonated chicken eggs worldwide. The need to push the development of new vaccine technologies, including recombinant approaches (e.g. VLP) is evident.

There are many gaps in our understanding of how and why influenza viruses cause disease and what influences disease severity. But there also are major gaps in our understanding of the efficiency of immune defenses against influenza infection, and the role of natural and cellular immunity in individual protection against severe disease. This could have a major application for vaccine design, e.g. in orienting the design of new vaccines towards vaccine candidates able to elicit these types of immune responses, in addition to neutralizing antibodies.

Major issues to be addressed and topics of interest are:

1. Disease burden

1.1 Assessment of the clinical, social and economic burden of seasonal influenza in developing countries where there is little recognition of influenza and no programs to control it. Countries which have undertaken burden studies in recent years, such as Thailand and Hong Kong, could be of great help. The determination of monthly burden of disease will be of help to public authorities in implementing control measures at the most appropriate time of the year.

1.2 Use of disease burden data from the above examples together with cost-analyses to implement or expand influenza control measures. Special attention must be directed to at-risk groups, such as the elderly and adults with underlying medical conditions such as pulmonary or cardiac conditions. Attention should also be directed to young children, who are particularly susceptible to the virus and serve as a reservoir for transmission to adults: the concept of a 'children's influenza vaccine' is a priority area.

1.3 Development and standardization of rapid diagnostic tests to diagnose respiratory illnesses, including influenza virus subtypes and strains, to better determine influenza disease burden.

1.4 Evaluation of the role of secondary bacterial pulmonary infections, such as streptococcal or staphylococcal infections, in the burden of influenza.

2. Influenza vaccines

2.1 An increase in the global production capacity of seasonal and pandemic influenza vaccines through transfer of technology to under-resourced countries. Promoting the transfer of manufacturing procedures which do not rely on chicken eggs would be of great advantage. The use of adjuvants with a dose-sparing effect should also be investigated in seasonal vaccines, as they could potentially increase the number of vaccine doses that can be prepared from a given amount of viral hemagglutinin and elicit broader protection. The safety of adjuvanted vaccines needs to be carefully addressed, especially in pregnant women and young children.

2.2 Development of new influenza vaccine formulations including "universal" vaccines that would elicit broad protection against a variety of influenza strains belonging to different clades and, if possible, different subtypes. The role of cellular immunity in broadening vaccine-induced protection should be investigated. The development of influenza vaccines based on, or containing, conserved viral proteins (e.g. M2, NP) should be continued and their potential interest confirmed. The development of new influenza vaccine types, such as VLPs or live recombinant vaccines, should be encouraged. The duration of protection elicited by influenza vaccines should also be carefully assessed.

2.3 Development of standardized reagents and assays to determine immune correlates of protection, including virus microneutralization tests, neuraminidase assays and cell-mediated immunity assays.

2.4 Thorough assessment of the safety of the different influenza vaccine formulations, whether live or inactivated (with or without an adjuvant), especially in young infants and pregnant women.

2.5 Exploration of possible correlates of protection, other than those linked to circulating antibodies, in human vaccinees.

3. Public Health issues

3.1 Development of public health policies and programs to reduce the impact of seasonal epidemic and pandemic influenza in developing countries through immunization and other public health measures. Major attention should be directed to under-resourced countries with no or little access to vaccines and insufficient health care infrastructures.

3.2 Development of best practices and methodology to evaluate the implementation of these policies and to measure their effectiveness in the control of seasonal epidemic and pandemic influenza.

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