

Community-acquired pneumonia: paving the way towards new vaccination concepts

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Abstract

Despite the availability of antimicrobial agents and vaccines, community-acquired pneumonia remains a serious problem. Severe forms tend to occur in very young children and among the elderly, since their immune competence is eroded by immaturity and immune senescence, respectively. The main etiologic agents differ according to patient age and geographic area. *Streptococcus pneumoniae*, *Haemophilus influenzae*, respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV-3) are the most important pathogens in children, whereas influenza viruses are the leading cause of fatal pneumonia in the elderly. Effective vaccines are available against some of these organisms. However, there are still many agents against which vaccines are not available or the existent ones are suboptimal. To tackle this problem, empiric approaches are now being systematically replaced by rational vaccine design. This is facilitated by the growing knowledge in the fields of immunology, microbial pathogenesis and host response to infection, as well as by the availability of sophisticated strategies for antigen selection, potent immune modulators and efficient antigen delivery systems. Thus, a new generation of vaccines with improved safety and efficacy profiles compared to old and new agents is emerging. In this chapter, an overview is provided about currently available and new vaccination concepts.

Introduction

The mucosa of the human respiratory tract represents a primary target for a large number of microbial pathogens. Typically, colonization is an asymptomatic process, resulting from the interplay between bacterial factors and host clearance mechanisms. Clinical illness may result from either the local release of bacterial toxins or the systemic dissemination of the pathogen after breaching the mucosal barrier. In the course of respiratory infections adaptive immune responses could be significantly impaired. This might lead to more severe forms of disease or to super-infections, which in turn complicate the clinical management of the patient. The most severe forms of respiratory infection tend to occur in very young children and among the

elderly, in whom immune competence is eroded by immaturity or immunosenescence, respectively. In addition, patients who are immunocompromised, as a result of disease or therapeutic interventions, have the greatest risk of developing a fatal infection.

Despite the availability of new antimicrobials and effective vaccines, community-acquired pneumonia remains a common and serious illness. In fact, it is a leading contributor to the nearly 4 million deaths occurring each year due to respiratory infections, especially in children from developing countries [1, 2]. The main causative agents of pneumonia differ according to the patient age and the geographic area. In addition, there are relatively few comprehensive studies on the specific aetiology of pneumonia [2] due to (i) overlaps in the clinical manifestations of the different syndromes, (ii) difficulties in establishing the precise aetiology, and (iii) frequent occurrence of co-infections. However, *Streptococcus pneumoniae*, *Haemophilus influenzae*, respiratory syncytial virus (RSV), and parainfluenza virus type 3 (PIV-3) have been identified as the main agents responsible for acute respiratory infections in children, whereas influenza virus related pneumonia is the leading cause of disease-related deaths in the elderly. In addition, the availability of new and more sensitive diagnostic tests have contributed to the identification of hitherto unknown lower respiratory pathogens, such as the human metapneumovirus (hMPV) and novel coronaviruses causing the severe acute respiratory syndrome (SARS).

Significant efforts have been invested in the last two decades to develop new diagnostic tools, to elucidate the molecular mechanisms of microbial pathogenesis and to understand host clearance mechanisms. This resulted in an improved knowledge on host responses to infection and immuno-pathogenesis, which in turn have facilitated the establishment of new prophylactic and therapeutic interventions. However, despite our accomplishments in vaccine development, there are many pathogens for which vaccines or adequate therapies are not available or the existent ones are suboptimal.

The main approach applied for vaccine development has radically changed in recent years. Whole cell vaccines are systematically being replaced by subunit vaccines, in which purified antigens or their coding genes are exploited in combination with new adjuvants and/or delivery systems. As a result, many of the vaccines under development will exhibit consistently improved stability, safety and efficacy profiles. They will also be amenable for mucosal administration, thereby mimicking natural infections.

Currently available vaccines

Influenza vaccines

Influenza A viruses are the most commonly responsible for severe respiratory illness in humans, followed by influenza B. The population's susceptibility

to infection is renewed annually, because of the rapid antigenic variation of this virus. The antigenic variation is due to the accumulation of point mutations in the two major surface glycoproteins of the virus, haemagglutinin (HA) and neuraminidase (NA). This can lead to an antigenic drift of the virus, which often leaves current influenza vaccines outdated and ineffective. Antigenic shift can also occur due to the segmented nature of the viral genome that favours the emergence of re-assorted strains, in which an entire glycoprotein can be acquired from a different animal influenza virus. Both types of variation represent a critical bottleneck for the establishment of a robust vaccination strategy against influenza. In fact, when an influenza virus with the capacity to spread from person-to-person and a complete new glycoprotein subtype suddenly emerges, a worldwide pandemic outbreak can result [3].

Inactivated vaccines against influenza

The earliest vaccines against influenza were whole cell vaccines obtained in the 1940s by inactivating viruses grown in the allantoic cavity of embryonated chicken eggs with formalin. While contemporary inactivated influenza vaccines are still produced in embryonated eggs, improvements in manufacturing have resulted in a highly purified and less-reactogenic detergent-split product. Three viral strains are selected on the basis of the previous year's surveillance data on the most prevalent subtypes, therefore, vaccine composition may vary from year-to-year. Vaccination has a high benefit:cost ratio, since influenza-related illness (e.g., hospitalizations and deaths) are effectively prevented [1].

The world's total vaccine production is approximately 300 million doses, with a maximum capacity of 900 million doses. However, the World Health Organization (WHO) estimates that there are about 1.2 billion people at high risk for severe influenza outcomes (e.g., elderly over 65 years of age, infants, health care workers, children and adults with underlying cardiopulmonary disease). Furthermore, the global infrastructure would not be able to handle the timely manufacturing and distribution of a vaccine for a pandemic outbreak [4]. One alternative would be to lower the quantity of antigen per dose and add adjuvants to the vaccine formulation, but this needs to be tested in clinical trials [5]. Another solution would be to improve current vaccine production technologies (i.e., egg-derived vaccines). However, there is the limited number of egg producers and viral strains can emerge, which could not be easily adapted to embryonated eggs. To overcome these problems, several pharmaceutical companies have embarked themselves on projects for the development of vaccines produced by growing the virus on cell lines. The influenza virus can be adapted to grow on a variety of mammalian cell lines, including Vero, PER.C-6, and Madin-Darby canine kidney (MDCK) cells [6–8].

This strategy would also improve the possibility of up-scaling vaccine production in face of a pandemic spread. Alternatively, it would be possible to develop a vaccine against any influenza virus, such as the avian H5N1 strain, by using reverse genetics techniques [9] (see below in advances in vaccinology).

Live attenuated vaccines against influenza

Cold adaptation was found to be a reliable and efficient procedure for the derivation of live attenuated viral vaccine strains for humans. Cold-adapted (*ca*) virus strains can grow in primary chick kidney cells or embryonated eggs at 25–33°C, however, they exhibit a reduced replication at 37°C. The process of genetic re-assortment with the transfer of the six internal genes from a stable attenuated *ca* master donor strain of influenza A or B to the new prevailing wild-type epidemic strain has been used to generate attenuated cold-reassorted vaccines with the proper level of attenuation, genetic stability and immunogenicity, which show low or absent transmissibility [10]. MedImmune and Wyeth have developed along these lines a trivalent live *ca* vaccine (Flumist) for intranasal spray delivery, which was licensed in 2003. In contrast, to parenterally-administered vaccines, this formulation triggers immune responses resembling those observed after natural infections [11]. Despite the moderate hemagglutination-inhibiting antibody titres observed in vaccinees, Flumist showed 92% efficacy over a 2-year period in children, including protection against antigenic variants that circulated during the second year [12–14]. This *ca* vaccine also stimulated the production of nasal IgA, as well as T-cell and interferon responses [15]. The cell-mediated immunity against virus matrix and nucleoprotein antigens may favour viral clearance and early recovery from illness [3]. The Advisory committee on Immunization Practices has recommended its use only in persons from 5 to 49 years of age, since side-effects were observed in young children (wheezing, nasal congestion) and there are no data available for elderly [16, 17]. Despite its remarkable genetic stability, this vaccine has to be kept at –18°C. Thus, a new heat stable derivative has recently been developed, which showed good efficacy in clinical trials [1]. A live vaccine based on a master virus strain developed at the Institute of Applied Microbiology (Austria) by growing wild influenza virus in Vero cells at 25°C was also demonstrated to be safe, well-tolerated and immunogenic after intranasal immunization in young adults [18].

Subunit and DNA vaccines against influenza

A number of subunit- or DNA-based vaccines are also in various stages of development. An influenza vaccine formulated in virosomes has been

commercialized by Berna Biotech (Inflexal[®] V); it contains the surface spikes of the three currently circulating influenza virus strains inserted in vesicle membranes of the three corresponding virus types (for more details see section “Pseudoviruses as antigen delivery systems”) [19]. This company has also developed a virosome-based nasal formulation. However, it was withdrawn from the market due to the presence of side-effects (i.e., Bell’s palsy), which was assumed to be linked to the presence of the *Escherichia coli* heat labile toxin (LT) as adjuvant. Two companies, Yeda and BionVax, are also developing a peptide-based influenza vaccine for nasal administration, which showed protective efficacy in humanized mice [1]. A subunit vaccine containing recombinant HA protein produced using a baculovirus system was successfully tested in a phase II trial in 64 to 89-year-old volunteers. An epidermal DNA-based influenza vaccine, which contained the HA gene from A/Panama/2007/99 delivered by particle-mediated epidermal delivery was also tested in humans by PowderJect [20]. Serum haemagglutination-inhibition antibody responses were observed in volunteers receiving a single dose of 1, 2 or 4 µg of DNA, with the strongest and most consistent responses in subjects vaccinated with the highest dosage.

Some immunization approaches aim at the development of a universal vaccine with a broad spectrum of protective activity against different influenza strains [21]. Among them, the use of the highly conserved trans-membrane M2 protein of the virion can be mentioned. A recombinant particulate vaccine has been engineered by genetically fusing copies of the M2 to the hepatitis B core antigen (HBc). The M2-HBc fusion protein spontaneously assembled into virus-like particles (VLP), which provided complete protection against a lethal challenge with influenza virus A in mice [22, 23]. Promising results were also obtained after vaccination with a M2 peptide conjugated with a *Neisseria meningitidis* outer membrane protein complex (OMPC) in monkeys [24].

Vaccines against the parainfluenza virus

The human PIV (hPIV) consist of four serotypes, with hPIV-3 being the second leading cause of bronchitis and pneumonia in infants. No vaccine has been licensed to date against PIV, however, several approaches are currently under investigation. The initial attempts to provide protection by using vaccines based on formalin-inactivated viruses failed. Subsequent work demonstrated that the glycoproteins haemagglutinin-neuraminidase (HN) and F, which are responsible for virus attachment and fusion, are able to stimulate the elicitation of neutralizing antibodies in animals. However, their poor immunogenicity in naïve subjects led to the currently favoured approach, which is based on the use of live attenuated PIV.

Live attenuated PIV vaccines have been developed from both human and bovine strains, which are amenable for delivery by the intranasal route. Candidate vaccines should be able to replicate and induce a protective immune response in young infants, even in the presence of maternally acquired antibodies. Two main attenuated strains have been studied in detail. One is the hPIV-3 strain *cp45*, which was selected after 45 passages of the virus in African green monkey cells at low temperature. The other is a bovine PIV (bPIV)-3 strain, which is antigenically related to the hPIV-3, and replicates poorly in humans. Both *cp45* and bPIV-3 have been evaluated in phase I/II trials in sero-positive and sero-negative children and in young infants. They were found to be over-attenuated in sero-positive children, but immunogenic in sero-negative children and infants [25]. However, the magnitude of the anti-HN response was lower in children who received the bPIV-3 vaccine [25]. This prompted the engineering of chimeric bovine/human PIV-3 candidates (e.g., hPIV-Nb strain in which the human nucleocapsid is replaced by the bovine counterpart, or a bPIV-3 strain that expresses the F and HN proteins of hPIV-3). Attenuated, chimeric viruses that contain PIV-3 *cp45* internal genes with the F and HN genes from either PIV-1 or PIV-2 have also been tested in hamsters [26]. Berna Biotech is also developing a virosomal formulation of the PIV-3 [1].

Vaccines against the respiratory syncytial virus

Using the successful approach of the influenza vaccine, a formalin-inactivated candidate against the respiratory syncytial virus (RSV) was tested in children in the 1960s. The consequence was the hospitalization of 80% of vaccinees and two deaths [1]. Moreover, vaccinated children also suffered more severe disease on subsequent exposure to the virus, as compared to unvaccinated controls [27]. This demonstrated that the elicitation of a strong immune response is not sufficient to confer protection against disease, and can even lead to immuno-pathological reactions. Thus, it is essential to stimulate the “right” type of immune response. In the particular case of RSV, host responses play an important role in the pathogenesis of the disease, thereby making the development of a preventive vaccine extremely difficult. In addition, naturally acquired immunity to RSV is neither complete nor long-lasting, and recurrent infections often occur [28]. However, older children and adults are usually protected, suggesting that protection against severe disease develops after several consecutive infections. Passive immunization with RSV-neutralizing immune globulins was also shown to prevent RSV infection in newborns with underlying cardiopulmonary disease [29]. This demonstrates that antibodies play a major role in protection against this disease, whereas T-cell immunity targeted to internal viral proteins appears to contribute to clearance.

Subunit vaccines against RSV

Although live attenuated vaccines seem to be preferable for immunization of naïve infants, subunit vaccines may be of choice for elderly, high-risk children and pregnant women. Candidate subunit vaccines based on purified F proteins (PFP-1, -2 and -3) were demonstrated to be safe and immunogenic, even during pregnancy [30]. Maternal immunization using a PFP-based vaccine could be an interesting strategy to protect infants younger than 6 months of age [25]. However, no significant protection was reported in a phase III trial performed on children 1–12 years of age with cystic fibrosis after vaccination with a subunit vaccine based on PFP-3 [31]. A formulation based on surface glycoproteins F and G together with the virion matrix protein M from RSV-A was tested in healthy adult volunteers in the presence of either alum or polyphosphazene as an adjuvant. Short-live neutralizing antibody responses to RSV-A and RSV-B were detected in 76–93% of the vaccinees, suggesting that annual boosting will be needed [32–34]. The central domain of the G protein of RSV-A is relatively conserved among viruses from the groups A and B. Thus, a recombinant vaccine candidate, BBG2Na, was developed by fusing the G2Na domain to the albumin binding region of streptococcal protein G. This candidate was shown to be moderate immunogenic in adult human volunteers, but its clinical development was interrupted due to the appearance of purpura in vaccinees [1].

Live attenuated vaccines against RSV

The main two difficulties associated with the generation of live attenuated vaccines against RSV are over- or under-attenuation of the virus and limited genetic stability. Temperature-sensitive (*ts*), *ca* and cold-passaged (*cp*) mutant viral strains have been generated. Despite the attenuation shown in adults and sero-positive children, *cpts* mutants still caused moderate congestion in the upper respiratory tract of sero-negative infants (1–2 months old) [35]. Recombinant RSV vaccines with deletions in non essential genes (e.g., SH, NS1 or NS2), which also carry *cp* and *ts* mutations in essential genes are currently being evaluated [1]. Through recombinant DNA technology chimeric viruses were engineered, which contain the genes of hPIV-3 surface glycoproteins F and NH together with those of RSV glycoproteins F and G in a bPIV-3 genetic background. One of these candidates was found to be attenuated and able to induce the elicitation of immune responses against both hPIV-3 and RSV in rhesus monkeys [36]. Similarly, a bPIV-3 genome was engineered to express hPIV-3 F and HN proteins and either native or soluble RSV F protein [37]. The resulting strain, which induced RSV neutralizing antibodies and protective immunity against RSV challenge in African Green monkeys, needs to be tested for safety and efficacy against RSV and PIV-3 in infants.

Vaccines against the severe acute respiratory syndrome (SARS)-associated coronavirus

This emerging disease was originally described in the Guangdong province of China in 2002. Even when the global outbreak of SARS was under control in 2003, new infections were reported in persons who had contacts with animals in 2003 and 2004 [38]. The typical SARS-CoV-like virus is not transmitted from animals to humans. However, under certain conditions the virus can evolve into the early human SARS-CoV, which has the ability to be transmitted from animals to humans or even humans to humans, thereby leading to localized outbreaks of mild disease. The early human SARS-CoV, under selective pressure in humans, may further evolve into the late human SARS-CoV, which can cause local or global outbreaks of typical SARS [39].

SARS can be easily grown in cell cultures [38]. Thus, there is an urgent need for vaccines, not only to prevent naturally occurring epidemic outbreaks, but also as a tool against the threat of biological weapons. Several structural proteins are expressed by SARS-CoV, including nucleocapsid, envelope and spike (S) proteins [38]. The latter is a type I trans-membrane glycoprotein, which is responsible for virus binding, fusion and entry, and being the major target of neutralizing antibodies [38, 40]. The extracellular domain of the S protein consists of two subunits, S1 and S2 [40]. The S1 subunit possess a receptor-binding domain (RBD), which is responsible for viral binding to one of its receptors [41, 42]. Vector-based vaccines expressing the S protein, as well as DNA vaccines encoding full-length S protein have been assessed in preclinical studies [43, 44]. When modified vaccinia virus Ankara (MVA) coding for full-length S protein was administered by either intranasal or intramuscular route, neutralizing antibodies were elicited [45]. However, vaccination of ferrets resulted in liver damage after challenge, raising some concerns about the safety of this approach [46].

Vaccines formulated using different synthetic peptides encompassing linear B cell epitopes from the S protein, which were identified using sera from convalescent patients, stimulated high antibody titres. Nevertheless, none of them triggered the elicitation of neutralizing activity. On the other hand, some studies demonstrated that although antibodies against S protein of the late SARS-CoV (Urbani strain) exhibit neutralizing activity, they can also enhance infection by an early human SARS-CoV isolate (GD03T0013) and the civet SARS-CoV-like viruses. A derivative of the S protein with a truncation at amino acid (aa) 1153 fails to cause antibody dependent enhancement of infection, but retains the ability to induce neutralizing antibodies. These findings suggest that the elimination of the putative heptad repeated 2 (HR2, aa 1153-1194), which is implicated in viral fusion, might abrogate the stimulation of virus infection-enhancing antibodies [47, 48]. The use of the nucleoprotein of the coronavirus in a DNA vaccination protocol also led to the stimulation of a protective response [49]. In contrast, protection

was not achieved when a recombinant PIV-3 expressing the nucleoprotein alone or together with the matrix protein was used [50]. This demonstrates that the selection of the delivery system and immunization strategy play a critical role in vaccine efficacy.

Vaccines against adenovirus

The human adenoviruses are divided into six subgroups (A–F). The adenovirus can cause large-scale epidemics of acute respiratory disease, and dissemination is especially favoured under conditions in which persons are housed communally. The subgroup A viruses, such as Ad31, have been associated with pneumonia in immunocompromised patients. Neutralizing antibodies directed against the capsid (hexon and fiber proteins) seems to be the main effector mechanism to prevent re-infections by adenovirus [3]. Until 1998, military recruits in USA were administered enteric-coated capsules containing live viruses from the serotypes 4 and 7. The virus, which was not attenuated if delivered by respiratory route, was able to replicate in the gastrointestinal tract without causing disease, thereby stimulating protective responses in the respiratory tract [51]. When the vaccine went out of production, outbreaks of respiratory diseases caused by adenovirus re-emerged among the military recruits [3]. Since serotypes 1, 2, 3 and 5 cause the 80% of adenovirus associated respiratory disease in young children, the development of a tetravalent vaccine similar to the above mentioned might solve the problem in children [52]. However, the implementation of a vaccine (live or attenuated) against adenovirus should be carefully evaluated, since recombinant adenoviruses are proposed both as vaccine vectors and as tools for the transfer of foreign genes in gene therapy protocols.

Vaccines against Streptococcus pneumoniae

Polysaccharide-based vaccines against *S. pneumoniae*

In 1945, MacLeod et al. [53] reported the protective efficacy of a capsular polysaccharide (PS) vaccine in military personnel during an outbreak of pneumococcal pneumonia. The immunization with purified PS showed a drastically reduced reactogenicity, in comparison with the previously used inactivated whole cell vaccines. This was a major breakthrough, not only in terms of safety, but also because it demonstrated that a specific virulence factor can be purified and effectively implemented for the prevention of an infectious disease, thereby paving the road for modern non toxoid-based subunit vaccines.

Although the serological correlates of immunity are poorly defined, type-specific anti-capsular antibodies are responsible for protective immu-

nity. However, immunity is serotype specific, rendering extremely difficult the development of a universal vaccine. This is in part due to the elevated number of serotypes, the regional variations in dominant serotypes and the lack of updated sero-prevalence data for certain regions. These problems have been partially solved by the use of PS-based polyvalent vaccines. The currently licensed formulations contain 23 serotypes of *S. pneumoniae*, which cover approximately 90% of serious pneumococcal disease, but only in Western industrialized countries. Relatively good antibody responses (60–70%) are elicited in healthy adults 2–3 weeks after a single intramuscular or subcutaneous immunization [54]. Unfortunately, they are poorly immunogenic in children aged less than 2 years, in immune compromised individuals (e.g., AIDS patients) and in elderly people with concomitant disease, and they do not induce good immunological memory. Randomized controlled trials in healthy elderly and young men also failed to show a beneficial effect against pneumonia [55]. However, vaccination is recommended for healthy people over 65 years of age to confer protection against invasive disease [54]. PS-based vaccines can be also used in pregnant women to stimulate the production of antibodies, which are transferred to the foetus *via* the placenta or to the newborns by breast-feeding. However, it is still a matter of controversy whether maternal vaccination can indeed protect newborns against pneumococcal infections [56].

Conjugate vaccines against *S. pneumoniae*

The second generation of PS-based conjugate vaccines stimulates stronger antibody responses, even in infants, young children and immune deficient individuals, as well as immunological memory. These vaccines also suppress nasopharyngeal carriage of the pathogen and reduce bacterial transmission in the community leading to herd immunity, which adds considerable value to their implementation. The introduction of these vaccines in USA in 2000 resulted in a dramatic decline in the rates of invasive pneumococcal disease [1, 57, 58]. A significant reduction in the incidence rates among non vaccinated individuals was also observed as a result of herd immunity [59, 60]. However, the licensed seven-valent vaccine does not contain some of serotypes that cause severe disease in developing countries (i.e., serotypes 1 and 5). New conjugate vaccines including more serotypes, such as the nine-valent vaccine (Wyeth) and two 11-valent vaccines (GlaxoSmithKline and Sanofi-Pasteur), should provide better serotype coverage.

Protein-based subunit vaccines against *S. pneumoniae*

New approaches to develop protein-based subunit vaccines against *S. pneumoniae* are currently being pursued by different research groups. This is

expected to enable the generation of a universal vaccine conferring protective immunity against a large number of serotypes, as well as to avoid the complexity of manufacturing a conjugate vaccine [61]. There are different pneumococcal candidate antigens, such as the pneumolysin, neuraminidase, autolysin, pneumococcal surface protein A (PspA) and adhesin A (PsaA), which are in an early phase of clinical development [1]. In addition, several promising candidates have been identified, which are currently being tested in pre-clinical experimental models [1]. Among them, the two iron uptake ABC transporters of *S. pneumoniae* (PiaA and PiuA), which trigger protective immunity against invasive pneumococcal disease in mice. Through the screening of *S. pneumoniae* genomic expression libraries with sera from convalescent patients, bacterial surface proteins were identified (e.g., BVH-3 and BVH-11) that promote the elicitation of protective anti-pneumococcal antibodies in mice [1]. A recombinant hybrid protein, BVH3/11V, has successfully been tested in toddlers and elderly volunteers. This candidate vaccine should be able to trigger serotype-independent responses, since the BVH3 and BVH11 antigens are common to all serotypes of *S. pneumoniae*.

Vaccines against typeable and non typeable Haemophilus influenzae

Conjugated *Haemophilus influenzae* type b vaccines

The major obstacle for developing an effective vaccine against *H. influenzae* capsular PS was related to the inherently poor immunogenicity of this T-cell-independent antigen. Antibody responses against PS are age-related, with extremely poor immunogenicity in infants during the first 18 months of life. Unfortunately, this age group exhibits the highest risk for invasive infections caused by *H. influenzae*. A PS-based vaccine against the *H. influenzae* type b (Hib) was licensed in the United States in 1985, for children more than 18 months old [62, 63]. The protective efficacy after licensure studies showed the inefficacy of this vaccine not only in infants, but also in older children [64]. This problem was solved by the generation of a conjugate Hib vaccine. To this end, the Hib PS (i.e., polyribosylribitol phosphate; PRP) was covalently linked to an immunogenic carrier protein, thereby leading to T-cell-dependent responses against the PS. Different conjugate Hib vaccines currently exist. These vaccines are HbOC, PRP-T and PRP-OMP, which make use of the mutant diphtheria toxin CRM197, the tetanus toxoid and the outer membrane protein from group B *N. meningitidis* as carriers, respectively. All of them trigger similar immune responses at the recommended doses. However, the dynamic of the elicited response may vary for each of them [65, 66].

Efficacy studies of these vaccines showed that they confer protection not only against meningitis, but also against pneumonia [67–69]. Although

Hib vaccines are highly effective, their cost is still prohibitive for the world's poorest nations. However, with the establishment of the Global Alliance for Vaccines and Immunization (GAVI), we have moved consistently ahead in making them also available for developing countries. GAVI has approved the establishment of a Hib initiative to support countries wishing to sustain Hib vaccination, as well as those exploring whether their introduction could be considered a priority in the near future.

H. influenzae typeable and non typeable: vaccination perspectives

Although the introduction of conjugated PS vaccines has significantly decreased the prevalence of invasive Hib disease, paediatric infections due to non typeable *H. influenzae* (NTHi) are still highly prevalent. NTHi is most often associated with otitis media, sinusitis and bronchitis. In addition, NTHi is an important cause of lower respiratory infection in adults with chronic obstructive pulmonary disease (COPD). Thus, the development of a vaccine against NTHi is considered an important goal in public health. In contrast to Hib, vaccines against the non-encapsulated NTHi strains must be directed against alternative virulence factors. The lipoproteins D and P6 are widely distributed and antigenically conserved among *H. influenzae* strains, and also trigger the elicitation of protective immunity in animals vaccinated by mucosal route [70–73]. Thus, their incorporation in vaccine candidates might facilitate the generation of a universal vaccine against all typeable and non typeable *H. influenzae*.

Vaccines against Bordetella pertussis

Even in the age of vaccine availability, *B. pertussis* continues to be a major cause of childhood morbidity and mortality (i.e., approximately 50 million cases and 300,000 deaths occur annually worldwide). Since the late 1940s, the incidence of whooping cough has dramatically decreased in most developed countries, as a result of widespread immunization. The first vaccine formulations, which are still in use, consist of preparations based on killed *B. pertussis*. The frequent incidence of minor adverse effects (e.g., fever, protracted crying and local erythematous reactions), as well as concerns raised by reports of serious neurological side-effects, resulted in a decline in vaccine acceptance and use [74]. This in turn led to a re-emergence of whooping cough and its complications. This serious problem prompted the development of a new generation of acellular vaccines.

In 1981 Japan was the first country to successfully introduce acellular vaccines against whooping cough in its immunisation programme [75], leading to a consistent reduction in the reported side-effects. In the mid 1980's a major phase III trial of acellular vaccines was undertaken in Sweden, at a

time when the banning of the whole cell vaccine had resulted in a pertussis epidemic in that country [76]. The first vaccine trials contained chemically detoxified pertussis toxin (PT) and filamentous haemagglutinin (FHA), or detoxified PT alone. The results of these trials showed that whilst producing good antibody responses, the vaccines failed to give an adequate level of protection in infants. The mono-component vaccine conferred no protection against infection, whereas the use of the two component candidate only gave incomplete protection against infection [77]. The results obtained in Japan and Sweden stimulated vaccine companies in the USA and Europe to establish vigorous research programmes aimed at the development of a new generation of acellular vaccines with higher efficacy. Currently available vaccines have incorporated chemically or genetically inactivated PT and additional virulence factors, such as FHA, the outer membrane protein pertactin (PRN) and fimbrial proteins (FIMs).

The efficacy studies of this second generation of acellular vaccines have demonstrated that they confer levels of protection equivalent to the whole cell vaccines. The advent of improved techniques for antigenic characterisation and the introduction of acellular vaccines containing genetically defined components also resulted in a reduction of lot-to-lot variation in comparison with conventional whole cell vaccines and the acellular formulation originally introduced in Japan. However, despite the wide implementation of vaccination campaigns in infants and children, the disease continues to be endemic. In addition, in countries with high vaccine coverage we are now observing a consistent increment in the cases of pertussis in adolescents and adults [78–80]. These patients can then transmit the disease to infants, thereby now representing a primary reservoir for bacterial transmission and cycling in the community.

The above-mentioned observations can be explained by one or more of the following factors: (i) improved detection techniques, (ii) major awareness on the possibility that bacteria may affect these age groups, (iii) vaccine-driven antigenic changes in circulating isolates, and (iv) reduction in vaccine efficacy over time. In this context, concerns have been raised about genetic variation between the strains used for vaccine preparation and circulating isolates. This seems to be true, since the currently used whole cell and acellular vaccines are prepared with strains that were isolated before mass vaccine introduction and show clear mismatches with respect to circulating strains. There is a steady tendency to decrease diversity in recent isolates, together with clonal expansion during epidemic outbreaks [81, 82]. Over time, at least two surface proteins (PT and PRN) may have changed sufficiently to allow for an increase in the incidence of disease. Unfortunately, our global information on antigenic variation and disease in adults and adolescent is extremely limited. Thus, despite widespread introduction of pertussis vaccines, it is essential to continue surveillance studies and collection of circulating strains. The present view is that successful control of pertussis in the community may require routine immunization of adolescents and adults

with the new acellular vaccines, perhaps in combination with the diphtheria and tetanus toxoids (DTaP). This intervention might help in turn to reduce the burden of disease and transmission to infants.

Vaccines against Chlamydia pneumoniae

Chlamydia pneumoniae is an intracellular bacterium transmitted person-to-person *via* respiratory droplets. This pathogen is a common cause of pneumonia, with infections usually being oligosymptomatic or asymptomatic in young age groups. However, the rate of asymptomatic carriage in the normal population is unknown. There is also a tremendous gap in our understanding of host response to infections caused by *C. pneumoniae*. Most of the studies have been focused on the development of efficient diagnostic methods. However, less work has been done on vaccine development, and there is a paucity of knowledge on the microbial components which may serve as target antigens. In fact, at present there are no licensed vaccines against *C. pneumoniae*. However, the potential of different antigens, such as the major OMP2 [83] have been assessed in experimental animal models. Nevertheless, mice vaccinated with OMP2 using a protocol based on priming with DNA and boosting with recombinant VLP showed only partial protection [84]. Recent studies also suggested that CTL responses play a role in protection and clearance [85]. Animals immunized with a mini-gene encoding seven H-2(b)-restricted CTL epitopes fused to a endothelial reticulum-translocation signal showed protection following intranasal challenge with a virulent *C. pneumoniae* [85].

The current view is that multi-component vaccine will be required in order to induce a protective response [86]. Using the promising approach of reverse vaccinology combined with proteomics (see section “Reverse vaccinology”), the whole-genome of *C. pneumoniae* was screened searching for vaccine candidate antigens among exposed and immune accessible surface proteins [87]. The selected candidates were then expressed in a heterologous system and used in immunization studies. Approximately 53 proteins were able to trigger the elicitation of *C. pneumoniae*-binding antibodies. When tested in secondary screenings, six of them were also able to neutralize bacteria *in vitro*, and four inhibited systemic dissemination of *C. pneumoniae* in a hamster model [86].

Vaccines against Moraxella catarrhalis

Moraxella catarrhalis is the third most common bacterial etiologic agent of otitis media in children. Furthermore, *M. catarrhalis* is an important cause of respiratory infections in patients with COPD. Thus, different studies have been carried out to characterize potential protective antigens. In

this context, two major OMP (CD and E) have been identified, which are considered prime candidate antigens for vaccine development. These proteins are expressed on the surface and show a high degree of conservation among circulating strains. Both OMP triggered the elicitation of bactericidal antibodies and protective immunity in preclinical models [88]. Additional candidates are the UspA1 protein [89], which seems to be required for bacterial colonization of the human upper respiratory tract, the iron-induced OMP B1 and LBP, and the iron-repressed OMP B2 [90]. A conjugate vaccine based on detoxified lipo-oligosaccharide was also tested in mice by intranasal route with encouraging results [91, 92]. Some of these candidates are planned to be tested in clinical studies soon [90].

Vaccines against Mycoplasma pneumoniae

Mycoplasmas are commensal microorganisms, as well as opportunistic pathogens. *Mycoplasma pneumoniae* is one of the causative agents of acute and chronic human respiratory diseases and the main responsible for primary atypical pneumonia, accounting for approximately 20–30% of all community-acquired pneumonia [93]. There is a considerable underreporting for *M. pneumoniae*-associated diseases. This is in part due to the wide diversity of clinical manifestations, the difficulties associated with its cultivation from clinical specimens and the lack of adequate diagnostic tools. No vaccines are currently available against this pathogen. However, studies conducted in human volunteers in the late 1960s demonstrated that a formalin-inactivated whole cell vaccine and an acellular extract were able to confer moderately protective immunity against *M. pneumoniae* [94]. Unfortunately, immune pathological reactions were observed following challenge with live organisms. Therefore, studies are still needed to understand the underlying mechanisms to the observed autoimmune responses [95]. More specifically, we need to elucidate the specific role played by humoral and cellular response in protection against *M. pneumoniae*. *M. pneumoniae* is one of the smallest self-replicating prokaryotic pathogens (approximately 800 kb). The complete genome sequence is now available. This is expected to expand our knowledge on the physiological and virulence properties of this agent, as well as new hints for vaccine development.

Vaccines against Legionella pneumophila

A previously unrecognized bacterium was isolated after the outbreak of Legionnaires disease in 1976, which was designated *Legionella pneumophila* [96, 97]. The spreading of *L. pneumophila* is increasing due to the use of air-conditioners and humidifiers, since infections can occur by inhalation of aerosolized contaminated water sources. Several approaches have

been developed in the fight against this facultative intracellular pathogen. Infection and immunization induce a rapid increase of antibody titres. However, antibodies do not seem to play a significant role in host resistance, particularly after aerosol challenge [98–100]. Some authors also suggested that these antibodies can promote bacterial phagocytosis, thereby favouring invasion and subsequent intracellular replication [101]. In contrast, cellular responses appear to be important for protection. Different vaccine candidates were tested in the past. Heat-, acetone- and formalin-killed *L. pneumophila* vaccines were not able to confer protective immunity in guinea pigs, whereas animals immunized with *L. pneumophila* membranes survive an aerosol challenge with virulent bacteria [98, 99]. Additional work demonstrated that also purified antigens, such as the major secretory protein [98], the major cytoplasmatic membrane protein [102], the peptidoglycan-associated lipoprotein [103], OmpS [104] and flagella [100] can confer protection against challenge with virulent *L. pneumophila*. Finally, different live attenuated mutants of *L. pneumophila* were used in animal infection models with promising results [105].

Vaccines against Pseudomonas aeruginosa

Cystic fibrosis (CF) patients are particularly susceptible to severe bacterial infections of the lung, being *Pseudomonas aeruginosa* one of the most prominent etiologic agents. Thus, significant efforts have been invested to develop a vaccine against this pathogen. Surface PS are among the antigens that were most intensively assessed. Berna Biotech have developed an octavalent vaccine against the eight most prevalent serotypes based on O-PS conjugated with the exotoxin A [106–113]. A consistent reduction in the number of CF patients with chronic *P. aeruginosa* lung infection was observed in a cohort receiving the basic immunization protocol, followed by yearly boosters over a period of 10 years [112, 113]. The conjugate vaccine induced the production of specific IgG antibodies and increased the number of IgG memory B cells. It is still unclear if cellular responses might contribute to the overall protection conferred by this vaccine. However, strong proliferative responses of lymphocytes with a Th1 phenotype were observed in vaccinated individuals in response to the carrier exotoxin A protein [113]. Alternative vaccination strategies are currently being tested in clinical trials. Among them, formulations based on a fusion protein between the outer membrane proteins F and I, which have been administered by parenteral and mucosal routes [114, 115]. These formulations were demonstrated to be safe in volunteers and conferred increased protection against *P. aeruginosa* in CF patients. Cell-surface alginate, flagella, components of the type III secretion system, inactivated toxins and proteases are other proposed target antigens [116]. Some of them are already in clinical trials alone or in combination [116].

New advances in vaccinology

When Pasteur returned from his summer holidays in 1881 to continue with his studies on chicken cholera, he inoculated chickens with an old culture of *Pasteurella multocida*, which was left during the whole summer on his bench. The animals that received the preparation were protected against a challenge performed with a fresh isolate. Thus, Pasteur developed the hypothesis that pathogens could be attenuated by exposure to environmental insults (e.g., high temperature, oxygen and chemicals) [117]. The strategy was then successfully extrapolated for developing anthrax vaccines in livestock in the 1880s, with significant economic benefits. This was followed by the generation of attenuated vaccines against rabies and other important pathogens towards the end of the nineteenth century. Pasteur's approach for "attenuating" or "inactivating" a pathogenic organisms still constitutes a cornerstone in vaccine technology [117]. This exemplifies that until recently the major achievements in vaccinology have been facilitated by technological (e.g., adjuvants, delivery systems, reverse vaccinology, genetic engineering) rather than immunological advances [117–119]. However, it is expected that the impressive knowledge accumulated in recent years in the fields of immunology, immune pathology and microbial pathogenesis will pave the road to a new golden era in vaccinology, in which knowledge and technology will enable rational vaccine design.

New technologies and approaches for vaccine development

Reverse vaccinology

In the 20th century, pertussis vaccines progressed from crude bacterial preparations to the highly purified antigens used for acellular vaccines. A similar quantum jump in technology allowed the development of subunit vaccines against influenza, Hib and *S. pneumoniae*, as well as the production of antigens by recombinant DNA techniques (e.g., genetically inactivated PT). Despite the fact that these techniques enable the production of almost any foreseeable antigen, the identification of suitable targets still remained as a main bottleneck for vaccine development [120].

The advent of genomics and its exploitation in the vaccinology field have rendered possible the implementation of a systematic and holistic approach for the screening, identification and prioritisation of candidate antigens. This new approach, called "reverse vaccinology" [121], does not require cultivation of the original pathogen, thereby being amenable for highly-pathogenic or non culturable micro-organisms. It is possible to predict and select the most promising candidates by the analysis of genomic sequences *in silico*, which will then be cloned and expressed in heterologous systems. The resulting proteins are then used to perform immunological and/or

functional studies to select the most promising candidates (e.g., able to induce the production of microbicidal or neutralizing antibodies, capacity to confer protective immunity). Flanking studies are usually carried out, such as molecular epidemiological analysis to assess their degree of conservation among circulating strains, or transcriptional profiling to evaluate their expression during natural infections [122].

The time-consuming process in which highly expressed components of an *in vitro* cultivable organism are identified (one at a time) and separated (different components between them) is one of the disadvantages that reverse vaccinology has solved. The conventional method usually requires 15–20 years to arrive to a clinical trial, whereas reverse vaccinology reduces the process to approximately 5 years. Reverse vaccinology also allows the identification of hundreds of potential candidates in a few days, in comparison with the small number of antigens that conventional approaches have provided after decades of research. Moreover, reverse vaccinology offers the possibility to select potential candidates independent of their expression levels or purification easiness.

The reverse vaccinology approach has proved its usefulness in the field for both viral and bacterial pathogens (e.g. hepatitis C virus, Group B meningococci, group B streptococci) [123, 124]. Reverse vaccinology has also become an essential tool for several vaccine development projects against agents causing community-acquired pneumonia (e.g., *C. pneumoniae*, *streptococci*). The potential and speed of genomic-based approaches was also shown when the nucleotide sequence of the coronavirus causing SARS was made available in less than one month. In addition, the increasing number of available genomes from bacteria and viruses would allow comparative genomic studies, thereby providing hints on conserved protein families and/or functional domains. This would facilitate the generation of vaccines using immunogens covering multiple micro-organisms [125]. Despite the incredible potential of reverse vaccinology, this approach also has some important limitations (Tab. 1). Among them is the fact that it is not possible to identify non-protein antigens (e.g., PS, glycolipids), which are the cornerstone for many successful vaccines (e.g., pneumococcal and Hib vaccines).

Reverse genetics

Currently available influenza vaccines (see above) are based on inactivated viruses, and, more recently, attenuated *ca* viruses and virosomes. All these vaccines exploit the same starting material (wild-type virus), which is inactivated or attenuated. The last approach consists in the co-infection of chicken eggs with the new isolate and a master attenuated strain, and subsequent selection for re-assorted viruses with the desired genotype/phenotype. However, the virulence of certain virus strains, such as the H5N1,

Table 1. Classical vaccine development *versus* reverse vaccinology

	Reverse vaccinology	Classical approach
Time required to reach the clinical phase	~ 5 years	~ 20 years
Type of organisms	Culturable and non-culturable	Only culturable
Antigens	Only proteins	Proteins, lipoproteins, polysaccharides and glycolipids
Genome	Necessary	Unnecessary
Target genes	All	Mainly <i>in vitro</i> expressed
Exclusion of known antigens	Yes	No
Need to handle microorganisms (e.g., highly pathogenic)	No	Yes
Surface and structural antigens	Yes (only proteins)	Yes
Internal antigens	Yes (only proteins)	Rarely
Antigens with low expression levels	Yes	Rarely selected
Number of candidate antigens	Many (more than hundred)	Few
Selection of antigens	Poorly or highly immunogenic	Mainly highly immunogenic
Antigens identification and separation	Easy	Could be difficult
Need for genetic tools during the initial discovery process	Not necessary	Usually essential (e.g., to create and complement mutants)
Need for genetic tools for antigen expression	Necessary for the initial phase of development	Only in a late phase of development

renders difficult the implementation of this traditional strategy. The use of reverse genetics represents a valid alternative for the generation of vaccines against RNA respiratory viruses, such as the influenza virus, PIV and RSV. It consists in the production of the virus from cloned DNA [126], thereby allowing the development of vaccines against any pandemic viral strain. In some cases (e.g., avian H5N1) an additional mutagenesis step would be required to attenuate its virulence [127]. Then, the new HA and NA segments would be transferred into an appropriate influenza A virus master strain adapted to grow in a cell line. The final re-assorted virus will have the antigenic specificity of the pandemic strain and the growth characteristics of the master strain [128, 129].

This technology would also allow production of the influenza vaccine in cells that are co-transfected with plasmids encoding for different frag-

ments of the virus [130]. Therefore, the complete genome is inside the cell and virus can be produced and assembled. One of the main advantages is that a plasmid encoding for HA and NA can be easily replaced. Therefore, re-assortment and selection become unnecessary. This method would considerably reduce the time for vaccine production, from many months to only a few weeks. Another advantage would be the simple manipulation of the genome (contained in plasmids), which would enable detoxification of specific virulence factors. Similar approaches can be implemented for other viruses, such as RSV, PIV and SARS-CoV. However, intellectual property and liability issues are still obstacles for the industrial development of reverse-genetics-based vaccines [131]. Furthermore, since the resulting viruses are considered genetically modified organisms, additional problems may arise from the regulatory stand point [131].

Mucosal delivery systems

Most of the infective agents are either limited to the mucosal membranes, or need to transit across them in order to cause disease. Therefore, it is highly desirable to elicit an efficient immune response at the local site in which the first line of defence is laid. The stimulation of a pathogen-specific response at the portal of entry is expected to impair infection (i.e. colonization), thereby reducing the risk of transmission to susceptible hosts. Parenterally administered vaccines mainly stimulate systemic responses, whereas vaccines given by the mucosal route mimic natural infections, thereby leading to efficient mucosal and systemic responses. Thus, there is a considerable interest in the development of mucosal vaccines. However, antigens administered by this route are usually poorly immunogenic. Different strategies are being pursued to overcome this bottleneck, among them can be cited the use of (i) advanced synthetic delivery systems, (ii) live attenuated bacterial or viral vectors, (iii) bacterial ghosts, (iv) pseudoviruses and (v) mucosal adjuvants [132–135].

Advanced synthetic mucosal delivery systems

Particulate antigens are more immunogenic than those in solution, due to their vulnerability to degradation by enzymes and extreme pH. Thus, it would be helpful to incorporate them into a protective vehicle. Often, these vehicles do not serve only to protect them, but can also enhance their uptake, promote targeting to antigen presenting cells and serve as adjuvants [136]. The most commonly exploited delivery systems are: (i) gelatine capsules, which are dissolved at alkaline pH in the intestine but not in the stomach, (ii) muco-adhesive polymers that are highly viscous inert PS, (iii) eldexomer and carboxymethyl cellulose, which have been used for oral, nasal and vaginal delivery, (iv) lipid-based structures with

entrapped antigens, such as immune stimulating complexes (ISCOMs) and liposomes, and (v) biodegradable micro/nano-spheres based on bio-compatible materials such as starch, copolymers of lactic or glycolic acid [137, 138]. Some of these approaches are currently being explored to develop vaccines against agents causing community-acquired pneumonia. Encouraging results have been obtained, among others, using surface antigens from *S. pneumoniae* encapsulated in micro-spheres [139] and a ISCOM-adjuvanted vaccine obtained by reverse genetics against the influenza virus, in preclinical models [140].

Live attenuated bacterial or viral vectors

Attenuated viruses and bacteria can be used not only as vaccine candidates *per se*, but also as delivery systems for heterologous antigens. Thus, many attenuated microorganisms have been exploited as a scaffold for the development of subunit vaccines against other agents, under the premise that the expression of the recombinant antigen(s) does not increase their pathogenic potential for humans or animals. The most frequently exploited bacterial vectors are attenuated derivatives of *Salmonella enterica* and *Shigella* spp., and the Bacille Calmette-Guérin (BCG). For example, vaccination with an attenuated *Salmonella* expressing the OprF-OprI was also shown to be able to confer protection against *P. aeruginosa* in a murine experimental infection model [141]. In addition, it was also demonstrated that a recombinant BCG-based vaccine expressing the PspA confers protection against *S. pneumoniae* in an infection animal model [142]. The use of commensals represents an alternative to attenuated organisms (e.g., lactobacilli). In this context it was demonstrated that oral administration of *Lactobacillus* expressing proteins from coronavirus can protect against a gastric infection [143]. Thus, this approach has been also proposed to combat SARS. Promising results were also obtained using x *Chlamydia psittaci* [144]. On the other hand, different attenuated viruses, such as MVA, bovine or attenuated hPIV-3 and adenovirus can be used as delivery systems for heterologous antigens [25, 145]. In fact, MVA has recently been exploited for antigens of the SARS associated coronavirus [146].

Bacterial ghosts

An alternative approach to the use of live attenuated carriers is given by the use of bacterial ghosts. Ghosts are generated by the conditional expression of the lethal lysis gene *E* from bacteriophage PhiX174 in Gram-negative bacteria [147–151]. This leads to the formation of a trans-membrane tunnel through the bacterial cellular envelope [147]. Due to the high internal osmotic pressure, the cytoplasm content is expelled through the tunnel, thereby leading to an empty bacterial cell envelope [152]. The presence of envelope components in the ghosts provides a strong danger signal through the activation of pattern recognition receptors [153]. In addition, bacterial

ghosts are efficiently taken up by antigen-presenting cells, stimulating their maturation and activation [154].

Bacterial ghosts retain all morphological, structural, and antigenic features of the cell wall and can be used as vaccine candidates *per se*. Ghosts can also be externally loaded with purified antigens. Alternatively, ghosts can be generated from recombinant bacteria expressing heterologous antigens, hence avoiding the difficulties associated with the purification steps. This technology also offers the possibility to manipulate the topology of the recombinant antigen (e.g., the antigen can be bound to the inner membrane, secreted into the periplasmic space or associated to the surface). Encouraging results have been obtained in preclinical models using ghosts expressing chlamydial antigens [135, 155].

Pseudoviruses as antigen delivery systems

Promising results have been reported using different types of pseudoviruses, such as virosomes and virus-like particles (VLP), which are non-replicating viral-like structures. Virosomes are based on the principle of reconstituting empty viral envelopes through integration of viral envelope proteins in liposomes. They offer the versatility of liposomes in terms of lipid composition, with the advantage of including viral membrane proteins. Virosomes are produced by disassembling the viral membrane envelope with detergents. Then, the viral nucleocapsid is removed by ultracentrifugation before reconstitution (Fig. 1). In contrast, VLP exploit the capacity of recombinant viral coat proteins to spontaneously self-assemble, thereby mimicking at structural level the viral capsid. VLP can be isolated after protein expression in eukaryotic cells or by *in vitro* assemblage from subunits produced in a heterologous system [156]. Their main advantages are the lack of the viral genetic material with an “intact” envelope, and the fact that they are significantly more immunogenic than soluble proteins. They can be used as vaccines *per se*, as well as a delivery system for protein- or nucleic acid based vaccines, or as carriers for small molecules. Foreign antigens can be expressed on their surface, or can be simply encapsulated. In addition, amphiphilic adjuvants can be incorporated into their membranes, thereby offering the advantage of combining an adjuvant and the antigen in one entity without a covalent attachment.

Pseudoviruses are especially attractive for mucosal vaccination protocols, since they offer the opportunity to use the natural route of transmission of the agents. Induction of serum antibodies, secretory IgA, T helper and CTL responses, and protection against mucosal pathogen challenge has been reported from studies in animals and humans [157–159]. The virosomes generated using the influenza virus retain membrane fusion properties very similar to the naïve virus. Therefore, they are able to deliver material to the cytosol of target cells, offering the possibility to access the MHC class I-restricted pathway of antigen presentation to prime CTL activity [160–162].

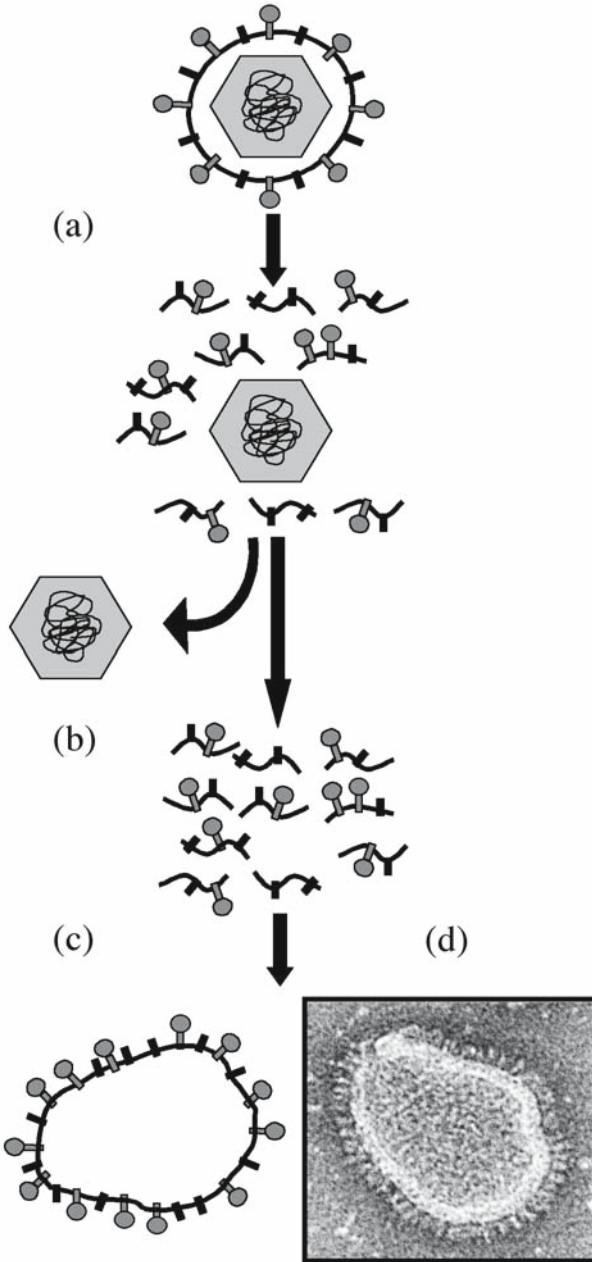


Figure 1. Virosomes are reconstituted viral envelopes, which incorporate the cell binding and fusion proteins of native virus without its viral genetic material. (a) Virosomes are produced by disassembling the viral membrane envelope with detergents. (b) The viral nucleocapsid is then removed by ultracentrifugation, and (c) they are reconstituted by removing the detergent with or without addition of lipids. (d) Electron-microscopy of an influenza virosome kindly provided by Etna Biotech.

Mucosal adjuvants

Bacterial toxins and their derivatives are among the first molecules that have been used as mucosal adjuvants. They are characterized by the presence of an A moiety with enzymatic activity, and a B moiety that mediates toxin binding to the target cells. Cholera toxin and the closely related *Escherichia coli* heat-labile toxin showed potent adjuvant activity when co-administrated with different antigens by the mucosal route [163–165]. However, their use in humans is hampered by their intrinsic toxicity. Thus, mutated derivatives were developed, in which the A subunit was modified to remove the ADP-ribosylating activity. The resulting polypeptides retain their adjuvant activity, in the absence of detectable toxicity [166–168]. However, additional studies have demonstrated that even these derivatives can lead to potential severe side-effects, such as retrograde homing of adjuvant and antigen to neural tissues [169]. This might explain, at least in part, the side-effects observed after intranasal vaccination against influenza with a virosomes-based formulation containing heat-labile toxin (i.e., Bell's palsy), which in turn led to its retraction from the market. However, chimeric derivatives lacking the targeting moiety for neural tissues (i.e., B subunit) are now available [170]. They might allow the exploitation of the high potential of these molecules for the development of vaccines against respiratory pathogens. In fact, preclinical studies provided the proof-of-concept for the usefulness of derivatives of bacterial toxins in the generation of acellular vaccines against microorganisms, such as *S. pneumonia* and *H. influenzae* [171, 172].

Other bacterial components were also explored for their activity as adjuvants. The monophosphoryl lipid A retains much of the immune stimulatory properties of LPS, without the inherent toxicity [165]. On the other hand, extracellular matrix binding proteins, such as the fibronectin binding protein I of *Streptococcus pyogenes*, also exhibit adjuvant activity [173]. This offers the possibility of using them as dual antigen/adjuvant moieties in the same formulation. Recent reports also demonstrate that vaccine formulations containing adamantylamide dipeptide, a non-toxic compound obtained by linking the L-alanine-D-isoglutamine residue of the muramyl dipeptide to the antiviral drug amantadine, confer protection against non typeable *H. influenzae* in preclinical models [73].

The innate immune system plays a critical early role in host defence against pathogenic microorganisms through the recognition of pathogen-associated molecular patterns [174]. This is achieved through the stimulation of pattern-recognition receptors (PRR) that sense a broad range of exogenous and endogenous danger signals [153, 174]. Toll-like receptors (TLR) represent the best-characterized family of PRR. Natural and synthetic TLR agonists are being used as immune modulators to optimize responses after vaccination. Since the identification of the TLR4, many mammalian TLR homologues have been identified (i.e., 10 in humans

Table 2. Toll-like receptors (TLR) and their ligands

TLR	Ligands
TLR1 (with TLR2)	Mycobacterial lipoprotein (LP), triacylated lipopeptides, lipoteichoic acid (LTA)
TLR2	LPS (<i>P. gingivalis</i>), fungal products (zymosan), peptidoglycan (PGN), LP, GPI anchors (<i>T. cruzi</i>), lipoarabinomannan, muramyl dipeptide
TLR3	Viral dsRNA, synthetic Poly (I:C)
TLR4	Gram-negative bacterial products, LPS, respiratory syncytial virus, synthetic lipid A, E5564, plant products, saturated and unsaturated fatty acids, murine β -defensin 2, BCG
TLR5	Flagellin
TLR6 (with TLR2)	<i>Mycoplasma</i> LP, LTA, PGN, diacylated LP
TLR7	GU-rich ssRNA, resiquimod, imiquimod
TLR8	GU-rich ssRNA, resiquimod, imiquimod
TLR9	Bacterial and viral DNA, unmethylated CpG-ODN
TLR10	Unknown
TLR11 (in mice)	Components from uropathogenic <i>E. coli</i> , and profiling-like from <i>Toxoplasma gondii</i>

and 13 in mice) [175]. Each TLR member binds specifically to different ligands (Tab. 2), alone or in combinations (e.g., heterodimers formed by TLR2 with either TLR1 or TLR6). An example of TLR agonist is bacterial DNA, but not vertebrate DNA, and synthetic oligodeoxynucleotides containing unmethylated CpG motifs. They act on TLR9, thereby inducing a strong Th1 responses by activation of dendritic cells [176, 177]. CpG motifs have been successfully used as adjuvants in preclinical studies of different candidate vaccines against agents causing community-acquired pneumonia [178-180].

Another important adjuvant with TLR-binding capacities is the *Mycoplasma*-derived macrophage-activating lipopeptide MALP-2, which act at the level of the TLR heterodimer 2/6 [181, 182]. MALP-2 promotes a global activation of cells from the innate and adaptive immune system [183, 184], such as macrophages, DC, T- and B-lymphocytes [183, 185]. When co-administered with an antigen by either the parenteral or the mucosal route, MALP-2 promotes the elicitation of humoral and cellular responses at systemic and mucosal level [186]. Preclinical studies suggested that MALP-2 could be exploited in vaccine formulations against the SARS-associated coronavirus, *M. catarrhalis* and influenza virus, among others (unpublished data).

DNA vaccines

DNA vaccination offers some advantage over the normal antigen vaccination, such as the fact that it is not necessary to express any antigen. In contrast, it is the biosynthetic machinery present in the cells of the vaccinees that takes care of this work. Furthermore, since eukaryotic cells are in charge of protein synthesis, their glycosylation and folding are optimal. However, the large-scale purification of DNA might be associated with high costs. This can be solved by the use of attenuated or inactivated bacteria or viruses as delivery systems [187]. This approach can also lead to an enhanced induction of antibodies, which is otherwise poor using conventional naked DNA vaccines. We have recently demonstrated that bacterial ghosts can be also exploited as a delivery system for DNA vaccines for both *in vivo* and *ex vivo* applications [188].

The potential of this approach is demonstrated by the fact that it is possible to optimize performance by a broad range of manipulations, such as (i) choice of optimal promoters, (ii) use of codon optimized genes for expression in mammalian cells, (iii) addition of nuclear localization signals or ubiquitination signals to improve expression and processing, and (iv) co-delivery of DNA constructs coding for immune modulatory molecules [189]. In addition, by the presence of immune stimulatory CpG motifs, the DNA vaccine constructs has built-in adjuvant properties. This vaccination approach is particularly suited for the stimulation of cellular immune responses [190]. Interestingly, several reports suggest that DNA vaccines may represent a valid alternative to prime the neonatal immune system, even in the presence of passive transferred maternal antibodies [191, 192]. In fact, promising results were also obtained in preclinical models of community-acquired pneumonia, such as influenza [193] and *S. pneumoniae* [194]. Furthermore, DNA coding for vaccine antigens appears to induce excellent immunological memory, which can be reawakened by later immunization or exposure to the pathogen.

New immunological concepts that need to be addressed to optimise vaccine design

The knowledge generated in several basic disciplines, such as immunology and microbial pathogenesis, has allowed the identification of critical bottlenecks for establishing a successful vaccination strategy. It is expected that in the coming years we will develop customized approaches to address each of them, in order to stimulate efficient protection against infective agents under specific clinical settings (i.e., newborns, aging individuals, immunocompromised patients).

The importance of immunological memory

B lymphocytes that have differentiated into plasma cells are the producers of antigen-specific IgG antibodies. Bone-marrow (BM) plasma cells have a short life, therefore, the BM reservoir needs to be replenished by the stimulation of memory B cells [195, 196]. The maximal life span of BM plasma cells is still debated. Only few factors have been identified that control the differentiation of antigen-specific B cells toward short- or long-life plasma cells or to memory B cells [119]. Beside the requirement of CD4⁺ T cells, the nature of the antigen [197] and the dose are also important. Higher antigen doses, as well as rapid vaccination schedules (closely spaced vaccine doses) tend to favour the rapid induction of short-term effectors, whereas lower doses of antigens preferentially support the induction of immune memory [198-201].

It was demonstrated that neonatal vaccination (priming) and infant boosting might be effective even when pathogen exposure occurs very early in life. In children in whom vaccine-induced Hib antibody titres have fallen to undetectable levels, memory is readily demonstrated [202]. However, immune memory *per se* is not enough to protect against pathogens that required high levels of neutralizing antibodies. The delay between memory B-cell reactivation and differentiation may limit the ability to interrupt pathogen invasion. Therefore, it is important to establish vaccination protocols in which the population is boosted at different ages in order to maintain the required levels of antibodies. This is particularly important in diseases in which antibodies play a central role in microbial clearance or toxin neutralization. In the particular case of community-acquired pneumonia, we should consider that aging individuals are neglected in many vaccination programs. However, the strategies proposed for elderly would be different from those used for small children, since the main factors affecting vaccine efficacy are immune senescence and immaturity, respectively. The attempts to give a rational solution to this issue are discussed in the next sections.

The immune system in children and elderly

The immune system in children

Immune responses to bacterial and viral antigens usually increase with age in a stepwise manner [203]. Prompt immunization after birth is required to induce active immunity against diseases that may occur early in life. Unfortunately, this strategy is limited by the relative immaturity of the neonatal and infant immune system. Some factors implicated in this poor response are the limited switch from IgM to IgG2 antibodies, impaired complement-mediated reactions and deficient organization of the splenic marginal zone. Vaccination studies performed in newborn mice suggested that limited germinal centre reactions may result from the delayed devel-

opment of follicular DC and limit plasma cell differentiation [204]. It was also showed that the neonatal BM has a limited capacity to support the establishment of long-life antibody-secreting plasma cells [205].

Thus, the responses to glyco-conjugates and to most T-cell-dependent antigens are usually affected [119]. Therefore, only few and highly immunogenic vaccines show significant protective efficacy after a single dose in infants. The limited IgG responses are extended all over the first year of life. In addition, the immune responses, particularly antibodies, elicited in the first year of life after vaccination rapidly decline [203]. However, the problem observed in infants in terms of magnitude and duration of immune response does not seem to affect efficient priming. In fact, the immune memory generated in neonates may be recalled later in life [119]. Nevertheless, strategies to generate strong and long-lasting protective responses in infants are still needed. This is in part due to the presence of maternal antibodies, which inactivate and clear the vaccine antigens, thereby rendering difficult the stimulation of an immature immune system [203]. In addition, the effects of adjuvants reported in adults cannot be extrapolated to neonates [206]. A potential strategy to overcome these problems would be to implement vaccination during pregnancy, to provide the required antibodies by placenta and later by maternal feeding [30, 207–209]. This could be complemented with an early priming of the “immature” immune system of the newborn by DNA vaccination, followed by a boost during the second half of the first year or later in life [203].

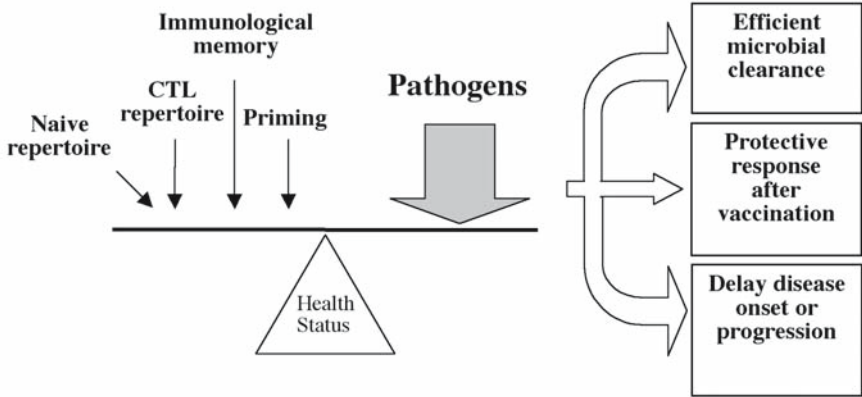
The immune system in the elderly

Poly-pathology and multiple organ failure is the rule rather than the exception in aging individuals. Thus, many systems are affected (e.g., endocrine, cardiovascular), and the immune system is not an exception. The mechanisms involved in the immune senescence process, which in turn may lead to poor response to vaccination, are not fully understood. However, it is clear that responses against certain vaccines are more affected by immune senescence than others (e.g., PS-based vaccines against *S. pneumoniae*) [210]. In contrast, the responses to a boost dose of the anti-tetanus vaccine are hardly affected by age [211].

A rapid decline of antibody responses, together with a relative restriction of the T-cell repertoire is characteristic of the immune senescence process. This restriction and the reduction in the pool of naïve cells can explain the poor CD4⁺ T cell responses against antigens that are cross-reacting with proteins which were seen earlier in life. In contrast, T-cells responses of healthy elderly individuals to new antigens are often unaffected. Nevertheless, the overall response to vaccination in the elderly is less efficient than in young adults, making more vigorous approaches necessary (Fig. 2).

In the case of influenza, the actual strategy is annual re-vaccination. However, there are concerns regarding the capacity to increase antibodies with proper specificity against re-assorted viruses in aging adults

Young adults



Aging individuals (immune senescence)

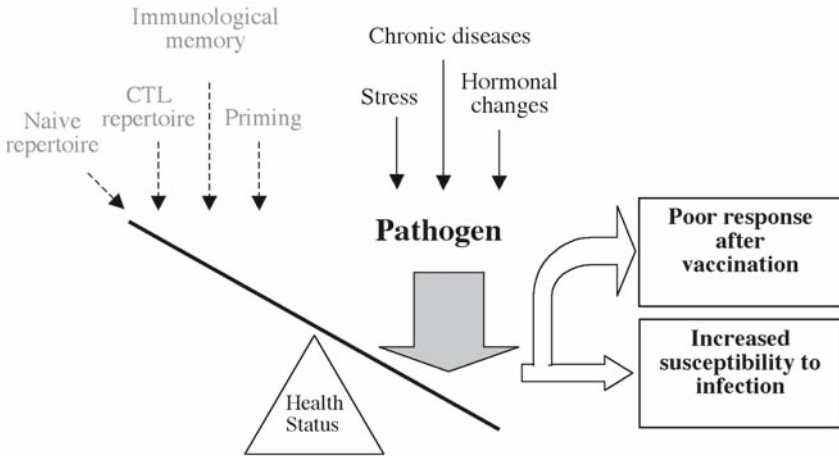


Figure 2. Factors affecting the responses in young adults and aging individuals after vaccination. The process of immune senescence impairs host response to both infection and vaccination. This critical issue needs to be considered during vaccine design and will require the development of special approaches.

who have been repeatedly infected or immunized. After exposure to a new, but cross-reacting antigenic variant, such individuals may respond by producing antibodies. However, these antibodies could be primarily directed against influenza strains, which were encountered earlier in life.

For example, individuals previously exposed to the “old” H1N1 influenza strain (i.e., 50 years ago), may respond differently from naïve adults who are vaccinated with a “new” H1N1 strain which have accumulated different mutations. The former might produce antibodies against the HA of the “old” H1N1 strain rather than to the cross-reacting epitopes of the new strain [212]. This is phenomenon is the so-called “original antigenic sin” [119]. On the basis of this observations, it was proposed that variations in vaccine efficacy might be due to differences in the antigenic distance between the vaccine strains and the epidemic strains responsible for influenza outbreaks [213]. However, this hypothesis was not confirmed by epidemiologic studies [214]. Even more, individuals aged 65 years or older who were annually vaccinated showed a significantly reduced mortality risk. Therefore, until now, it seems that the antigenic sin does not represent a major practical obstacle in influenza vaccination and additional strategies may not be required.

Concluding remarks

Despite the broad availability of vaccines against agents causing community-acquired pneumonia, they still represent an important cause of death, human suffering and economic losses. However, we have dramatically expanded our knowledge on the pathophysiology of diseases caused by respiratory pathogens, their virulence factors and the effector mechanisms responsible for their clearance. It is becoming clearer which microbial components are attractive as vaccine targets, as well as the type of immune response needed to confer protection against disease. Thus, it is now possible to address vaccine development using rational rather than empiric approaches. This is facilitated by powerful bioinformatics tools for the accurate prediction of epitopes and proteasome trimming [215–217], as well as by the availability of a broad palette of immune modulators and delivery systems. Therefore, we can predict that new and improved vaccines against the etiologic agents of community-acquired pneumonia will considerably reduce the global impact of this disease in the coming years.

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