The Grand Challenge for the Future
ed. by Stefan H.E. Kaufmann and Paul-Henri Lambert

Novel vaccination strategies

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Introduction

Vaccination is one of the most significant public health interventions ever made, sparing millions of people from infectious diseases. It has been calculated that use of currently available vaccines saves more than eight million lives annually, which corresponds to one person saved every five seconds. These remarkable results have been achieved in 200 years of vaccination history, which are largely based on two basic, simple technologies: the inactivation of toxins and pathogens with formaldehyde treatment (first performed by Glenny and Hopkins in 1923 and Ramon in 1924) [1–3], and the stable attenuation of pathogens by serial passage *in vitro*.

From 1920 to 1980, these now "easy technologies" were applied to develop vaccines that have been used worldwide to control or even eradicate many infectious diseases. One of the most brilliant successes of vaccination is the eradication of smallpox virus in 1977. It has been estimated that to date, smallpox eradication has spared the global community of some 350 million new smallpox victims and about 40 million deaths from the disease. The annual savings as a result of vaccination being stopped and hospitals being able to be converted to other uses is estimated to be in excess of US$ 2000 million each year [4].

Vaccination has also resulted in the elimination of poliovirus from the Americas in 1994, Western Pacific in 2000 and Europe in June 2002. The goal of the WHO Global Polio Eradication Initiative is to interrupt transmission of wild poliovirus by end-2004 leading to global certification by 2008. Overall, since the Initiative was launched in 1988, the number of cases has fallen by over 99%, from an estimated 350,000 cases to the 89 cases reported in the first three months of 2004. In the same period, the number of polio-infected countries was reduced from 125 to 6 [5].

Moreover, the incidence of seven other frightening diseases (diphtheria, measles, rubella, mumps, pertussis, *Haemophilus influenzae* type b – Hib –, and tetanus) has fallen by more than 98% in those states where the related vaccines have been introduced (see Tab. 1).

Table 1. Universal routine vaccination reduced by more than 98% the incidence of nine harmful diseases from the US and eradicated two of them (smallpox and polio).

Disease	Max number of cases (year)	Number of cases in 2002	Reduction (%)
Smallpox	48,164 (1901–1904)	0	100
Poliomyelitis	21,269 (1952)	0	100
Diphtheria	206,939 (1921)	1	99.99
Measles	894,134 (1941)	44	99.9
Rubella	57,686 (1969)	18	99.78
Mumps	152,209 (1968)	270	99.86
Pertussis	265,269 (1934)	9771	98.20
H. influenzae type b	20,000 (1992)	<100	98.79
Tetanus	1,560 (1923)	25	98.44

Source: CDC (1998) Impact of vaccines universally recommended for children – US, 1990–98. *MMWR* 48: 577-581; modified) [94].

Novel strategies for novel vaccines

The historical technologies for vaccine production have achieved remarkable successes, but the battle against infectious diseases has not been won. During the last several million years of existence on earth, microorganisms have learnt how to escape the surveillance of our immune system, either hiding themselves from host responses, or developing the capacity of infecting different hosts. These two main mechanisms of pathogen defense explain why vaccines against several pathogens such as HIV, HCV, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Plasmodium falciparum*, *Schistosoma* spp. and others are difficult to develop and are not yet available.

Moreover, during the last 25 years, there has been a big revolution in microbial ecology: pathogens that have circulated since many years, but whose existence was ignored were discovered (HCV, MetaPneumoVirus, etc.); old pathogens have changed their geographical habitat and have been introduced into new areas where they have become endemic (e.g. West Nile Virus in North America); completely new pathogens have emerged and pose a serious threat to human health (HIV, SARS Coronavirus, etc); pathogens thought to be controlled have re-emerged (*Mycobacterium tuberculosis*, etc.) (Tab. 2).

It was soon clear that the old vaccine strategies were unable to face with the new challenges posed by infectious diseases, and modern approaches were needed. Therefore, from the beginning of the 1980s, taking advantage of the better understanding of the immune system and the emergence of the recombinant DNA technologies, new vaccines were developed (Tab. 3). Moreover, nowadays the range of technologies and strategies available for vaccine development is extremely broad. The availability of the whole genomic sequences of many pathogens is considered a real revolution and

Table 2. New and emerging pathogens identified in the last 25 years

Period	Pathogen
1980–1990	HTLV HIV *Escherichia coli* O157H7 *Borrelia burgdorferi* *Helicobacter pylori* Human herpesvirus type 6 *Ehrlichia chalffeensis*
1990–2004	HCV *Vibrio cholerae* O139 *Bartonella* spp. Hantavirus Nipah virus West Nile virus TT virus Meta Pneumo virus SARS Coronavirus

a start of a new era in the vaccine field. Genomics, proteomics, molecular biology, recombinant DNA, and advanced immunology have removed most of the technical barriers that formerly limited vaccine development approaches to prevent and not only treat infectious diseases, but eventually also cancer, allergies, and other chronic conditions.

Conjugate vaccines

It is now common knowledge that the capsular polysaccharide of many important bacterial pathogens is their major virulence factor and that vaccination using purified polysaccharides could protect people from those diseases. Polysaccharide vaccines have been developed against *Neisseria meningitidis* serogroup A (MenA), group C (Men C), group Y (MenY), and group W135 (Men W135) in the late 1960s [6, 7], against *H. influenzae* type b in the 1980s [8], against 23 types of *Streptococcus pneumoniae* in the early 1990s [9], and against *Salmonella typhi* in the mid 1990s [10]. However, the use of all these vaccines is inadequate. Polysaccharides are T-independent antigens: their immunogenicity and efficacy is very poor or absent in infants, so that they are unsuitable to be included in the infant routine immunization schedule.

The solution was found by covalently linking the sugar to a carrier protein (conjugation). This procedure converts T-independent antigens into T-dependent ones by providing a source of appropriate T-cell epitopes (present in the carrier protein). Conjugation technology is one of the biggest achievements of the novel vaccination strategies. The capacity of transforming poor immunogens such as polysaccharides into excellent immuno-

Table 3. Vaccines licensed after 1980

Disease	Year
Hepatitis B (HBV)	1986
Haemophilus influenzae type b, conjugate	1990
Salmonella typhi (live attenuated, capsular polysaccharide)	1991
Hepatitis A (HAV)	1994
Varicella	1995
Acellular pertussis (aP)	1996
Influenza (adjuvanted)	1997
Lyme disease	1998
Neisseria meningitidis group C conjugated	2000
Streptococcus pneumoniae 7 valent conjugated	2000

gens such as the protein-sugar complex has provided outstanding tools for the fight against infectious diseases.

Haemophilus influenzae type b (Hib)

The first conjugate vaccine developed was the one against *Haemophilus influenzae* type b (Hib) [11]. Hib is a respiratory pathogen that causes a wide spectrum of human infections. Before the availability of the Hib conjugate vaccine, Hib was the leading cause of bacterial meningitis, especially in infants, in most developed and developing countries worldwide, resulting in substantial morbidity and mortality. The first Hib conjugated vaccine was introduced in the US in December 1987 for children 18 months of age and older; subsequently, more immunogenic vaccines were introduced, in US as well as in many other countries, in the early 1990s, for the use in infants from the age of two months.

Within a few years, the effectiveness and public impact of Hib conjugate vaccines on Hib disease has been demonstrated in all those countries that have introduced the vaccination [12]. In the US, the annual cases of Hib invasive disease among children under five years of age decreased from more than 20,000 of the pre-vaccination era [13], to less than 50 in 2002 [14].

In general, the decrease in incidence of disease has exceeded that predicted on the basis of estimated proportion of the population that is completely immunized, suggesting that the vaccine use is able to generate herd immunity. On this topic, many studies have shown that Hib conjugated vaccines are able to reduce nasopharyngeal carriage [15–18], which gives a major contribution to herd immunity. Precisely how Hib conjugate immunization reduces nasopharyngeal colonization, whether this impact is due to decreased acquisition or decreased duration of carriage is not clear. Additional work on the immunology and microbiology of transmission and carriage is needed to clarify this issue. On the other hand, reduction of Hib carriage may open ecological niches for *H. influenzae* non-type b strains

and therefore potentially increase the risk of colonization and invasive disease by other dangerous strains, for which a vaccine is not available [19–21]. However, so far, widespread vaccination against Hib has not caused this problem: strains recovered from vaccinated children are indistinguishable from those recovered from unvaccinated ones [22].

Streptococcus pneumoniae

The first conjugate vaccine licensed for human use, initially in US in February 2000, and subsequently in Europe, contains capsular polysaccharides of seven serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) conjugated to the CRM197 as carrier protein by reductive amination. Other vaccines under evaluation cover nine and 11 serotypes. However, due to practical and economic reasons, only a limited number of serotypes could be included in conjugate vaccine formulations [23]. The choice of the strains should be based to target the serotypes that most commonly cause diseases among population and the geographic region to be vaccinated [24]. The seven serotypes in the first licensed conjugate vaccine account for >80% of isolates from blood and cerebrospinal fluid of children aged <2 years in the US, compared with <60% of isolates from older children and adults in US, and with <60% of isolates from young children in Latin America and Asia [25, 26].

The seven-valent conjugate pneumococcal vaccine has been recommended in US for all infants as three primary doses (approximately at 2, 4, and 6 months of age) and one booster dose at the ages of 12 and 15 months [27]. This vaccine repeated the successes of the Hib conjugate vaccine, showing high efficacy in preventing meningitis and efficacy, although to less extent, in preventing pneumonia [28] and acute otitis media [29] due to the vaccine serotypes. Moreover, several studies have established that the conjugate vaccine is effective also in reducing nasopharyngeal carriage of the serotypes covered by the vaccine, potentially extending the benefits beyond those children who are vaccinated. Post licensure evaluation of the efficacy of the seven-valent conjugated vaccine found that the reduction in disease was greater than the percentage of children who have been vaccinated, with evidence of herd immunity [30].

Neisseria meningitidis serogroup C

Neisseria meningitis group C conjugate vaccines began clinical development at the end of the 1980s, and, after extensive clinical studies during the 1990s, were proved to be safe and capable of inducing highly bactericidal and boostable immune responses in infants and children [31–39]. These vaccines were first introduced in the United Kingdom in late 1999, to be used

for a countrywide vaccination initiative. Population-based active surveillance monitored age-specific and capsular group-specific incidence of disease and estimated vaccine coverage [40]. The results were impressive: within 18 months of vaccine introduction, the cases of MenC diseases almost disappeared in the vaccinated population, with an estimated vaccine efficacy of 90%, or above, in all targeted age groups [41]. According to a large carriage study performed in the United Kingdom, the vaccine induced also a 66% decrease in the carriage of MenC strain, with no evidence of any increase in carriage of MenB isolates or other capsular groups [42]. Moreover, the vaccine provides some evidence of herd immunity, as there is also a reduction of MenC disease in unvaccinated individuals ranging from 34 percent (9–14 years old) to 61 percent (15–17 years old) [43].

Since 1999, the MenC conjugate vaccine has been licensed in many European countries and also in Canada and Australia, either as routine infant immunization, or for catch-up programs of varying forms.

Multivalent meningococcal conjugate vaccines containing groups A, C, Y, and W135 are in clinical development. Introduction of these vaccines into routine infant and/or toddler immunization programs could have a substantial effect on the incidence of meningococcal disease [44, 45].

Recombinant DNA for subunit vaccines: HBV example

In the 1970s it was discovered that protection towards hepatitis B is correlated to the presence of antibodies against the surface antigen of the virus (HBsAg), which circulates in the bloodstream of infected subjects in large quantities. These discoveries led to the development of first generation, plasma-derived HBsAg subunit vaccines, first licensed in the US in 1982 [46]. Concerns about safety of blood products and the advances of recombinant DNA technology conducted to the development of second-generation recombinant subunit vaccines produced in yeast or in mammalian cells [47], which were first licensed in US in 1986. The application of the recombinant DNA technology has the advantage to produce safer vaccines than the plasma- derived ones and in unlimited supplies. This recombinant HBV vaccine is highly effective: a standard course of three doses induces protective levels of anti-HBs (i.e. >10 mIU/ml) in over 95% of healthy infants, children and adolescents and in more than 90% of healthy adults younger than 40 years. After the age of 40, immunogenicity drops below 90%: however, and by the age of 60 years, 65 to 75% of vaccinees develop protective anti HBs titers [48–50]. Moreover, the vaccine prevents development of chronic hepatitis in at least 75% of early-vaccinated infants born from HBeAg-positive mothers [51].

Cost-benefit analyses have strongly supported the introduction of universal immunization against HBV to newborns [52]. Since 1992, the WHO has called for all countries to add hepatitis B vaccine into their national

childhood immunization schedules, and substantial progress has been made in implementing this recommendation: by the end of 2003 more than 160 countries worldwide will have introduced the vaccine [53]. Substantial help in achieving this goal has been given by the Global Alliance for Vaccines and Immunization (GAVI) and the Vaccine Fund, which since 2000 began to provide technical and financial support to introduce routine HBV immunization into 71 of the world's poorest countries [54].

The effectiveness of routine infant HBV immunization in significantly reducing or eliminating the prevalence of chronic HBV infection has been demonstrated in a variety of countries and settings. The experience in Taiwan is particularly impressive. Universal vaccination decreased the prevalence of HBsAg carriage in children younger than 15 years of age from 9.8% in 1984 to 0.7% in 1999 [55]. Moreover, rates of hepatocellular carcinoma (HCC) among cohorts of children born after routine infant immunization have started in the mid 1980s declined by greater than 50% [56]. These results clearly show that the HBV vaccine is the first successful anti-cancer vaccine, opening the way to other anti-cancer vaccines like vaccines against human papilloma virus (HPV) that causes cervical cancer [57], or *Helicobacter pylori*, responsible for many gastric cancers [58].

Genetically detoxified vaccines

The first example of a vaccine developed with a new rational strategic design, taking advantage of the power of the molecular biology and genomic technologies is the recombinant acellular pertussis vaccine.

A vaccine composed of the whole, killed *Bordetella pertussis* cells has been available for mass vaccination since the late 1940s [59]. This vaccine is very efficacious in preventing the disease, but the presence of severe adverse reactions, although no proof exists of their having been caused by the vaccine, brought about a drop in vaccine compliance in the 1970s and stressed the need for a new safer vaccine.

A considerable amount of work was initially required to identify the bacterial antigens responsible for the induction of protection. Pertussis toxin (PT) was then identified as a major protective antigen, while other antigens, such as adenylate cyclase, filamentous haemagglutinin (FHA), pertactin and the fimbriae, were found to provide some help in protective immunity. Several researchers developed acellular pertussis vaccines containing purified PT inactivated by the old classical chemical treatment with formaldehyde and glutaraldehyde. However, it is recognized that this treatment of PT may be associated with significant reversion rates [60].

To overcome these problems, the pertussis toxin was detoxified by using the powerful tools of molecular biology to make stable changes, by site-directed mutagenesis, to the amino acids responsible for the toxicity of pertussis toxin. This molecular approach allowed the development of a natu-

rally nontoxic molecule, inactivated by a rational design that did not need a denaturing chemical treatment [61].

The genetically detoxified PT had successfully shown its safety and immunogenicity in clinical trials, both in adult volunteers and in infants and children, either as a monovalent mutant PT alone [62, 63], or in association with FHA and pertactin [64, 65], and also with FHA and pertactin in association with diphtheria and tetanus toxoids (DTaP) [66, 67]. These trials showed that the different acellular vaccine formulations containing the non-toxic PT mutant were extremely safe, and much safer than the whole-cell pertussis vaccines. Furthermore, all formulations induced high titers of anti-PT neutralizing antibodies and very strong antigen-specific T-cell proliferative responses. Interestingly enough, five to six years after the primary immunization schedule, this vaccine still exhibited an efficacy of about 80%, and both antigen-specific antibody and $CD4^+$ T-cell responses were still detectable at significant levels [68, 69].

The new strategy used to develop this genetically detoxified acellular pertussis vaccine shows that this approach can provide tremendous advantages, eliciting very high, protective immune responses with very low amounts of antigen.

The genomic approach: reverse vaccinology

Despite the introduction of these new strategies (conjugation, recombinant DNA etc.), one of the major issues for the development of new vaccines is the identification of protective antigens from a microorganism. Biochemical, immunological and microbiological methods have been successful in many cases, but they require the pathogen to be grown in laboratory conditions, are time-consuming and allow for the identification only of the most abundant antigens, which can be purified in quantities suitable for vaccine testing. Unfortunately, in many instances the most abundant proteins could not be good vaccine candidates. Moreover, the antigens expressed *in vivo* during the infection could not be expressed *in vitro* to the same extent. Furthermore, when dealing with non-cultivable pathogens, there is no approach to vaccine development.

The availability of the complete genome sequence of a free-living organism (*H. influenzae*) [70] marked the beginning of the "genomic era" that opened novel strategies in vaccine design. The possibility of determining the whole sequence of a genome (bacterial, viral or parasitic) led to the idea of using the genomic information to discover novel antigens that have been missed by conventional vaccinology. This approach, called "reverse vaccinology", involves the computer (*in silico*) analysis of the microbial genome sequence and predicts those antigens that are most likely to be vaccine candidates [71, 72]. The success of genomic-based strategies for vaccine development is highly dependent on the criteria used for the *in silico* selection of

the potential antigens, which are usually secreted or extracellular proteins, more easily accessible to antibodies than intracellular ones. Several approaches can be used to mine genomic sequences, and the appropriate combination of various algorithms and the critical evaluation of the information generated are essential for the proper selection of the antigens.

The *in silico* approach results in the selection of a large number of genes. Therefore, it is necessary to use simple procedures that allow large numbers of genes to be cloned and expressed, like robotics and PCR. The product of each PCR reaction is cloned and screened for expression in a heterologous system. Successful expression depends on the predicted localization of the protein. Integral transmembrane proteins have proven to be particularly difficult to produce by recombinant techniques in *E. coli*.

Once purified, the recombinant proteins are used to immunize mice and the post-immunization sera are analyzed to verify the computer-predicted surface localization of each polypeptide and their ability to elicit a quantitative and qualitative immune, protective response.

The genomic approach has many novel features and advantages. There is no need to cultivate the pathogen. As the whole process starts *in silico*, it could be equally applied to cultivable and non-cultivable microorganisms. Pathogens dangerous to handle can be studied as easily as commensals. Virtually all protein antigens encoded by the pathogen's genome could become vaccine candidates, regardless whether they are expressed *in vitro* or *in vivo*, abundant or scarce. This process allows the identification of all the antigens seen by the conventional methods and the discovery of novel antigens that work on a totally different paradigm.

Unfortunately, reverse vaccinology suffers from limitations related to our incomplete knowledge of vaccine immunology: a rate-limiting step of the process is linked to the rarity (or, more often, absence) of widely accepted correlates of protection and reliable animal models of infection. The other constraint of this approach is the inability to identify non-protein antigens such as polysaccharides, and CD-1 restricted antigens such as glycolipids, which represent new promising vaccine candidates.

Meningococcus B

Meningococcus B represents the first example of the application of the genomic approach and the demonstration of the power of this novel strategy for target antigen identification [73]. Conventional approaches to MenB vaccine development have been ineffective for more than 40 years. A capsular polysaccharide-based approach, which has been extremely successful for other major meningococcal serogroups, cannot be used for this strain, because the MenB capsular polysaccharide is identical to a widely distributed human carbohydrate (α(2–8)N-acetyl neuraminic acid, or polysialic acid). This condition not only makes the polysaccharide a poor

immunogen for humans, but also a potential generator of autoantibodies. On the other hand, a protein-based approach, developed using surface-exposed proteins contained in outer membrane vesicles (OMVs), has shown to have efficacy in clinical trials only against homologous strains, especially in children below five years of age, because of the high sequence variability of the major protein antigens [74].

Using the reverse vaccinology, DNA fragments were screened by computer analysis while the *N. meningitidis* sequencing project was in progress. The screening was aimed to select proteins predicted to be on the surface of the bacterium or to have homologies to known bacterial factors involved in pathogenesis and virulence. After clearance of cytoplasmic proteins and known *Neisseria* antigens, 570 genes predicted to code for surface-exposed or membrane-associated proteins were recognized. Successful cloning in *E. coli* and expression was achieved for 350 proteins, which were then purified and tested for localization, immunogenicity and protective efficacy in animal models. Of the 85 proteins found to be surface exposed, 22 were able to induce complement-mediated bactericidal antibody response, which is the most convincing indication of the capability of inducing protective immunity. In addition, to test the appropriateness of these proteins as candidate antigens for conferring protection against heterologous strains, the proteins were evaluated for gene presence, phase variation and sequence conservation in a panel of genetically diverse MenB strains representative of the global diversity of the natural *N. meningitidis* population. Most of the selected antigens were able to induce cross-protection against heterologous strains, demonstrating that these new antigens are good candidates for the clinical development of a vaccine against MenB [73, 75].

It is impressive to notice that within only 18 months the genomic approach allowed the discovery of more potential vaccine candidates than the previous 40 years of conventional research.

The success of the MenB project, together with the availability of an increasing number of genomic sequences and the faster and faster development of bioinformatics tools, has encouraged the application of the reverse vaccinology approach to many other pathogens. Nowadays, this approach is widely used to develop vaccine candidates against those pathogens for which conventional approaches have failed so far. The potential of the genomic approach is so extensive that it could be used not only for bacteria, but also for viruses and parasites.

Streptococcus pneumoniae

Despite the successes of the conjugate vaccine against *Streptococcus pneumoniae* (see above), the absence of cross protection between the different serotypes, with the threat of serotype replacement by strains that are not represented in the vaccine composition, makes it unsuitable for universal

use [20]. To identify more appropriate vaccine candidates, the whole genome sequence of *S. pneumoniae* was scanned and 130 potential ORFs with significant homology to surface proteins and virulence factors of other bacteria were identified [76]. 108 of these proteins were effectively expressed in *E. coli* and used to immunize mice; six of them were able to confer protection against pneumococcal challenge in a mouse sepsis model. Flow cytometry confirmed the surface localization of these new proteins. Furthermore, each of the six protective antigens showed a high degree of cross reactivity against the majority of capsular antigens that are expressed *in vivo* and are immunogenic during human infection, providing a good base for the development of improved vaccines against *S. pneumoniae*.

Streptococcus agalactiae

Streptococcus agalactiae, or group B streptococcus, is the leading cause of bacterial sepsis, pneumonia, and meningitis in neonates in US and Europe [77]. As for *S. pneumoniae*, the capsular polysaccharide is the major protective antigen, but at least nine different seroptypes exist with little or no cross protection [78]. Therefore, a protein-based vaccine is required to overcome serotype differences.

The complete genome of a serotype V strain of *S. agalactiae* was determined and analyzed [79]. The genome is predicted to code for 2175 ORFs of which 650 were predicted to be exposed on the surface of the bacteria. Approximately 350 of these ORFs were successfully expressed in *E. coli* and used to immunize mice. Using the sera in ELISA and flow cytometric analysis against intact bacteria, it has been demonstrated that 55 of these proteins are in fact measurably expressed on the surface of the bacterium. These new antigens are now being evaluated in *in vitro* and *in vivo* models for their capacity to protect against invasive infection by group B streptococcus.

Staphylococcus aureus

To identify vaccine candidates for *Staphylococcus aureus*, an approach based on genomic peptide libraries and well-characterized human sera was developed [80]. *S. aureus* peptides were displayed on the surface of *E. coli via* fusion to one or two outer membrane proteins (LamB and FhuA) and probed with sera selected for high antibody titers and opsonic activity. The exhaustive screening of the two different peptide expression libraries by the application of MACS technology (magnetic cell sorting) enabled the profile of antigens that are expressed *in vivo* and that are able to elicit an immune response in humans to be identified. A total of 60 antigenic proteins were identified, most of which were predicted to be secreted, or located on the surface of the bacterium, and their antibody-binding

sites were mapped. These antigens represent promising vaccine candidates for further evaluation.

Porphyromonas gingivalis

A process very similar to the one used to develop a vaccine against MenB was used also for *Porphyromonas gingivalis*, a pathogen implicated in the etiology of chronic adult periodontitis [81]. From a genomic sequence of 2000 genes, 120 were selected using a series of bioinformatics tools. The selected genes were cloned for expression in *E. coli* and screened by Western blotting using sera from human periodontitis patients and animal antisera. This subset of 40 recombinant proteins were then purified and used to immunize mice, which were subsequently challenged with live bacteria in a subcutaneous abscess model. Two of these recombinant proteins (showing homology to *Pseudomonas* sp. OprF protein) demonstrated significant protection in the animal model and therefore could represent potential vaccine candidates [82].

Chlamydia pneumoniae

Chlamydia pneumoniae is an obligate intracellular parasite with a complex biphasic lifecycle: an extracellular infectious phase characterized by a spore-like form, the elementary bodies (EB), and an intracellular replicating stage characterized by the reticular bodies (RB). The pathogen is a common cause of community-acquired acute respiratory infections and more recently has also been associated with atherosclerotic cardiovascular disease [83].

Because of the technical difficulties in working with *C. pneumoniae* and the absence of reliable tools for genetic manipulation, not much is known about the cell surface composition of the EB. To define the surface protein organization of *C. pneumoniae*, a systematic genomic and proteomic approach was used [84]. The approach is based on six main experimental steps: (i) *in silico* analysis of the *C. pneumoniae* genome sequence to identify genes potentially encoding surface proteins (including outer and inner membrane and periplasmic proteins); (ii) cloning, expression, and purification of selected candidates; (iii) use of purified antigens to generate mouse immune sera; (iv) analysis of sera specificity by Western blotting of total EB extracts; (v) assessment of antigen localization by FACS analysis on whole EBs; and (vi) identification of FACS-positive antigens on bidimensional electrophoresis (2DE) maps of *C. pneumoniae* EB proteins. The results of this systematic genome-proteome approach represent the first successful attempt to define surface protein organization of *C. pneumoniae* and raise the possibility to find suitable candidates for a purified vaccine.

Reverse vaccinology for bioterrorism-associated agents: Bacillus anthracis and Yersinia pestis

Currently licensed anthrax vaccines have been developed during the 1960s and are based on cell-free filtrates containing mainly the Protective Antigen (PA). PA is the common cell-binding domain capable of interacting with two different domains, the lethal and the edema factors, which elicit cell damage. These vaccines, originally licensed for selected veterinary personnel, textile workers and abattoir workers, have been widely used for the first time for the US military during the 1990–91 Desert Storm campaign. However, this extended use has raised issues and controversies regarding safety, efficacy, and an unsatisfactory dosing regimen, as the vaccine requires 6-dose series of injections and annual boosters [85, 86].

Therefore, identification of novel antigens is essential for the development of second-generation *B. anthracis* vaccines. ORF products similar to proteins involved in bacterial pathogenesis, and secreted and surface exposed proteins are the focus of the research. Using functional genomic analysis, 11 candidates were selected. In this case, a simple method that relied on the *in vitro* translation of the linear full-length DNA of the selected ORFs was used. Polypeptides obtained *in vitro* were then evaluated for immunogenicity by analysis of their reactivity with hyperimmune anti-*B. anthracis* antisera. The combination of bioinformatic genomic analysis and an efficient and fast screening facilitated the identification of unknown antigenic proteins, three of which appear to be similar to immunogenic PA. These new proteins could represent parts of a second-generation anthrax vaccine [87].

Old plague vaccines are based on killed whole cell preparations [86, 88]. Although they appeared to be effective against endemic plague, they were not considered to be protective against respiratory plague, which is the form of most concern in case of biological attack. Moreover, these vaccines have high rates of reactions, both systemic and local, especially with successive doses and suffer from short duration of protection, requiring biannual boosters. A live-attenuated vaccine (EV76 strain) also existed. While this vaccine seemed to be effective, it retained some virulence and in most countries it was considered not suitable for human use.

In a search for novel attenuated vaccine candidates for use against *Y. pestis*, a signature-tagged mutagenesis (STM) strategy was used [89]. A library of tagged mutants of the virulent *Y. pestis* Kimberley53 strain was generated. Screening of 300 mutants through two consecutive cycles resulted in selection of 16 mutant strains. Characterization of the phenotypes and genotypes of the selected mutants led to identification of virulence-associated genes coding for factors involved in global bacterial physiology (e.g., *purH*, *purK*, *dnaE*, and *greA*) or for hypothetical polypeptides, as well as for the virulence regulator gene *lcrF*. One of the avirulent mutant strains was found to be disrupted in the *pcm* locus, which is presumably involved in the

bacterial response to environmental stress. This Kimberley*53pcm* mutant was superior to the EV76 live vaccine strain in an animal model in which mice were infected subcutaneously with the virulent *Y. pestis* Kimberley53 strain, either because the mutant induced 10- to 100-fold-higher antibody titers to the protective V and F1 antigens and because it conferred efficacious protective immunity. These results indicate that mutants with mutations in the *pcm* locus can serve as a platform for generation of a novel live vaccine with more promise for inducing efficacious protective immunity to virulent *Y. pestis* strains than previously suggested mutants.

New strategies for new diseases: SARS example

The SARS epidemic has been the first infectious disease outbreak to fully benefit from the revolutionary technologies of the post-genomic era. Thanks to an unprecedented collaboration effort between scientists from many countries, coordinated by WHO and including clinical, epidemiological and laboratory investigations, within 15 days from the issue of the global alert, the causative agent of the new disease was discovered [90]. In less than a month after the initial identification of a new coronavirus (called SARS-CoV) as the infectious agent of SARS, two independent genome sequences of the virus had been obtained [91, 92]. Within three months, the genome sequences of 20 independent clinical isolates were made available in the GenBank database. The rapid availability of the genomic sequence of the new virus has been very important from the public health perspective, giving prompt answers to a number of critical questions. It was clear that the agent was a natural (and not a laboratory-fabricated) coronavirus, diagnostic kits were set up, and possible drug and vaccine targets identified.

Today, some of these vaccines, based on a killed virus, are already being tested in pre-clinical and even in clinical trials, but their efficacy needs to be shown. Moreover, in addition to the traditional approach, a number of newer strategies are being used. These include subunit vaccines containing recombinant spike protein expressed in mammalian cells or yeast, either alone or in combination with other SARS-CoV antigens. Alternatively, these antigens could be delivered by DNA immunization by non-replicating viruses, or viral vectors that are based on adenovirus, canarypox, modified vaccinia virus Ankara (MVA) or alphavirus. In particular, the development of non-replicating coronavirus-like particles that mimic the structure of native virions could prove promising in the search for a successful vaccine as they display a large repertoire of antigenic sites and discontinuous epitopes [93].

It is crucial to remember that none of these approaches would have been possible in such a way and with such a speed without the knowledge of the complete genomic sequence of the virus. To make a comparison, the

last time mankind faced the appearance of a new pandemic was in the early 1980s, with the appearance of HIV: at that time, only 20 years ago, it took two years to identify the etiologic agent of the infection, and a safe and effective vaccine is still lacking.

Conclusion

When Edward Jenner gave birth to the concept of vaccination little could he know that after more than 200 years mankind would still be battling against emerging and re-emerging infectious diseases. However, contemporary society is lucky in that science is constantly offering more effective tools to combat and control infectious diseases. The question is whether the many social, political, cultural, and economic barriers that still exist *vis-à-vis* vaccines will be eventually overcome, thus recognizing the real value of vaccination.

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