Hemagglutinin consensus-based prophylactic approaches to overcome influenza virus diversity

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Summary
Each year millions of people are infected by influenza viruses, and this causes a substantial economic and health burden on our society. Influenza epidemics and pandemics are attributable to the ongoing evolution of influenza viruses through antigenic drift and shift, respectively. One of the reasons for the continuous circulation of influenza viruses in the human population is the incomplete protection conferred by currently available seasonal influenza vaccines against possible arising drifted or shifted influenza strains. Recently, tremendous efforts have been focused on the development of a more effective broadly reactive or universal influenza vaccine. The main objective of underdevelopment vaccines is to protect the human population not only from currently circulating virus strains but also from possible future variants without the need for their continuous update. Different approaches have been developed to reach this goal and elicit an effective and cross-protective immune response. Among these, consensus-based prophylactic approaches to effectively prevent influenza infections are the major focus of this review.

Approcci vaccinali basati sulla sequenza consenso dell'emoagglutinina per contrastare la diversità del virus influenzale

Parole chiave
Virus dell'influenza, Vaccini universali per l'influenza, Sequenza consenso, *Computationally-optimized broadly reactive antigen* (COBRA).

Riassunto
I virus dell'influenza infettano ogni anno milioni di persone con un onere economico e sanitario sostanziale per la nostra società. Le epidemie e le pandemie di influenza sono attribuibili alla continua evoluzione dei virus dell'influenza, rispettivamente causate dal *"drift"* e dallo *"shift"* antigenico. Uno dei motivi che determina la continua circolazione dei virus influenzali nella popolazione umana è la protezione incompleta conferita dai vaccini influenzali stagionali attualmente disponibili contro possibili ceppi influenzali andati incontro a *"drift"* o *"shift"* antigenico. Di recente, grandi risorse sono state impiegate per lo sviluppo di un vaccino più efficace contro l'influenza, ampiamente protettivo o universale, con l'obiettivo principale di proteggere la popolazione umana non solo dai ceppi virali attualmente in circolazione ma anche da possibili varianti future, senza la necessità di un suo aggiornamento continuo. Per raggiungere questo obiettivo, ed ottenere una risposta immunitaria efficace e ampiamente protettiva, sono stati messi a punto diversi approcci: tra questi, al centro di questa review, ci sono quelli profilattici basati sulle sequenze consenso.

Introduction

Influenza virus infections can be categorized into two epidemiological forms: seasonal and pandemic (Paules *et al.* 2017).

Seasonal influenza epidemics are caused by influenza A and B viruses in humans, resulting in 3-5 million severe cases and 300,000-500,000 deaths globally every year. The economic burden of influenza virus-induced disease is close to $100 billion in the U.S. each year (Molinari *et al.* 2007).

Infection by a seasonal influenza virus results in fever, nasal discharge, coughing, sore throat, headache, myalgia, and nausea. In the case of a severe disease

and hospitalization, secondary bacterial infections or a disproportionate inflammatory response represent the most common cause (Erbelding *et al.* 2018).

Differently, influenza pandemics caused by influenza A emerge at unpredictable intervals and they cause significantly increased morbidity and mortality compared to seasonal influenza (Paules *et al.* 2017).

In the past century, four pandemic outbreaks have occurred: in 1918, 1957, 1968, and 2009 (Palese 2004).

Furthermore, in the past few decades, animal influenza viruses, such as H5N1 and H7N9 avian influenza, have caused sporadic human infections and deaths. Influenza infections with these viruses, termed pre-pandemic viruses, are originally zoonotic and do not demonstrate sustained person-to-person spread. However, viral mutations may occur, allowing efficient transmission among humans and possibly leading to the next influenza pandemic (Nicholson *et al.* 2003).

Despite the availability of a seasonal vaccine, influenza remains a public health concern. Each influenza season, a set of virus strains, representing one H1N1, one H3N2 and 1 or 2 influenza B isolates, are selected for the inclusion in the annual seasonal influenza vaccine. The effectiveness of this standard of care (SOC) influenza vaccine ranges between 10-60% (Caspard *et al.* 2017, Flannery *et al.* 2018). The occurrence of a possible mismatch between circulating strains and vaccine strains, due to ongoing antigenic changes (also known as drifts) is the main reason of a low prophylactic effectiveness (Osterholm *et al.* 2012). In fact, the influenza strains used in annual vaccine formulations are selected twice annually following the influenza seasons in the northern and southern hemispheres. For this reason, this strategy keeps us at least one year behind compared to the evolving circulating influenza viruses. Moreover, virus growth in eggs during vaccine production may allow for additional mutations, further contributing to the vaccine mismatch with the circulating strains (Zost *et al.* 2017).

Additionally, seasonal influenza vaccines do not provide protection against possible occurring pandemic strains. In fact, the outbreak of a novel influenza virus with a pandemic potential requires the development of a pandemic strain-specific vaccine.

For all these reasons, there is an urgent medical need to improve the efficacy of the current influenza vaccine to limit the public health consequences of both seasonal and pandemic influenza infections. To this end, influenza vaccines that are more broadly and durable protective are needed (Erbelding *et al.* 2018, Sautto *et al.* 2018, Sautto *et al.* 2019).

In this review, we describe hemagglutinin (HA) consensus-based prophylactic approaches that have been developed for the different influenza virus subtypes over the years with a particular focus on the computationally-optimized broadly reactive antigen (COBRA)-based methodology.

Consensus-based approaches and design of computationally-optimized broadly reactive antigens (COBRA)

In order to overcome antigenic drift and mismatch of current influenza vaccines for pandemic and seasonal viruses, different groups have designed and developed over the years different consensus-based approaches for the influenza virus (Giles *et al.* 2012, Castelli *et al.* 2013, Carter *et al.* 2016, Wong *et al.* 2017, Wu *et al.* 2017, Elliott *et al.* 2018, Stepanova *et al.* 2018, Tsybalova *et al.* 2018).

From a definition point of view, a protein-based consensus sequence encodes the most common amino acid at each position of a protein. Thus, in the context of a pathogen-related protein, the generation of a consensus sequence captures a sequence that is more relevant in a current epidemic outbreak.

It has been previously demonstrated that a consensus-based approach for the influenza hemagglutinin (HA) protein is effective in protecting from influenza A infection. Moreover, consensus-based prophylactic approaches have been also investigated as a strategy for eliciting broadly reactive immune responses against other viruses such as human immunodeficiency virus (HIV), chikungunya virus (CHKV) and hepatitis C virus (HCV) (Scholte *et al.* 2013, Meyerhoff *et al.* 2017, Tarr *et al.* 2018).

More in detail, a novel methodology of antigen design using multiple rounds of consensus building to generate candidates termed computationally optimized broadly reactive antigens (COBRAs), have been described (Giles *et al.* 2012, Carter *et al.* 2016, Wong *et al.* 2017).

In brief, the COBRA methodology starts from inferring the phylogenetic tree from HA amino acid sequences derived from different outbreak groups in a given influenza A subtypes. Primary consensus sequences are then generated for each outbreak group. Subsequently, secondary consensus sequences are generated for each subclade using the primary consensus sequences as input. The secondary consensus sequences are finally aligned and the resulting consensus sequences, designated COBRA, are generated (Figure 1) (Giles *et al.* 2012). Expression of this novel antigens is then required to assess the breadth of protection of the elicited immune response in vaccinated pre-clinical animal models.

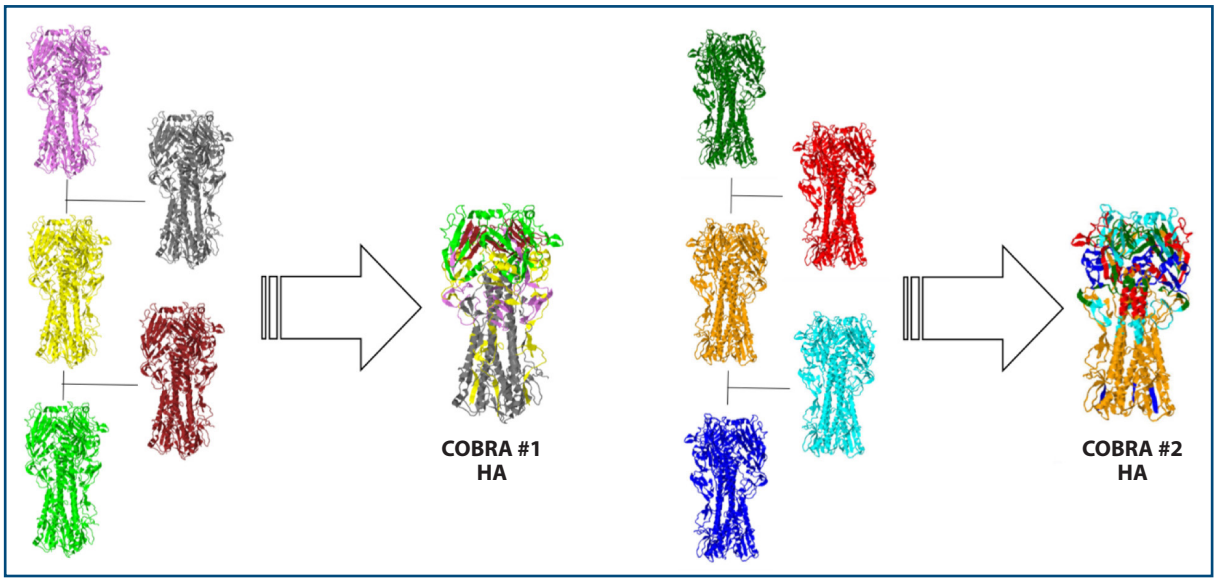

Figure 1. *Schematic representation of the COBRA strategy.* A phylogenetic tree is inferred based on included hemagglutinin (HA) amino acid sequences. As intermediate steps, primary and secondary consensus sequences are generated. Subsequently, the secondary consensus sequences are aligned and the resulting final consensus sequences, designated COBRA, are generated. Following their expression, COBRA candidates are then tested in animal models and the functional activity of the elicited immune responses is evaluated.

H5N1

Pandemic events of influenza are caused by the emergence of pathogenic and transmissible new viral strains to which there is a low or absent herd immunity in the population (Nicholson *et al.* 2003). Outbreaks of highly pathogenic avian influenza (HPAI) of the H5N1 subtype are of particular concern because of the high mortality rate (~ 60%) and pandemic potential, given the immunological naïve status of the human population (Cui *et al.* 2017). In order to develop a vaccine that elicits broadly reactive antibody responses against emerging H5N1 isolates, the development of consensus-based H5N1 immunogens has been described. Classical consensus-based approaches traditionally consist in aligning a population of sequences and selecting the most common residue at each position. These sequences are expected to effectively capture conserved linear epitopes and elicit cross-reactive cellular immune responses, especially for those antigens that are not expressed on the pathogen surface. These approaches have shown promising results, eliciting cross-reactive immune responses against different antigens including HA (Elliott *et al.* 2018), neuraminidase (NA) (Moise *et al.* 2013) and matrix (M1) proteins (Tsybalova *et al.* 2018). As an example, consensus-based H5N1 HA vaccines expressed from DNA plasmids elicit broad antibody responses (Laddy *et al.* 2007). However, the role of more conserved proteins in conferring protection, such as in the case of the M1 protein, is debated. In fact, the immune response targeting these antigens is correlated with a modulation of the disease instead of a proper protection (Sautto *et al.* 2018). Moreover, consensus-based antigen design is inherently influenced by the input sequences used to generate the synthetic molecule and as such is subject to sampling bias. In order to overcome this bias, the COBRA methodology was described to generate broadly protective H5N1 vaccine candidates (Giles *et al.* 2012). In particular, COBRA H5 immunogens were designed based upon HA amino acid sequences from clade 2 H5N1 human infections. These proteins were able to bind sialic acid, as well as mediate the viral particle fusion, confirming their retained functional activity (Giles *et al.* 2011). Vaccination of animal models with COBRA H5 immunogens, expressed on the surface of viral-like particles (VLP), elicited protective levels of antibodies endowed with HAI activity against representative isolates of each H5N1 subclade of clade 2 (Giles *et al.* 2012). Importantly, vaccinated mice and ferrets were completely protected from a lethal challenge with a clade 2.2 H5N1 virus and the same results were also confirmed in vaccinated nonhuman primates (Cynomolgus macaques) (Giles *et al.* 2012).

It is noteworthy that this breadth of protection can be potentially increased when using an oil-in-water emulsion formulation and can confer protection against challenge as efficiently as the homologous matched vaccine (Allen *et al.* 2017).

Finally, the immune responses elicited by the COBRA HA immunogens were compared to those elicited by a mixture (polyvalent) of different primary H5N1 isolates. Notwithstanding COBRA and polyvalent

vaccines protected vaccinated mice and ferrets from the challenge with highly lethal H5N1 influenza viruses, COBRA-vaccinated animals had higher-titer antibodies to a panel of H5N1 HA proteins, a decreased viral replication, less inflammation in the lungs of mice, and reduced virus recovery in ferret nasal washes. Furthermore, the fact that the serum from vaccinated mice protected recipient animals more efficiently than the serum obtained from polyvalent-vaccinated mice and that the cell-mediated immune response was comparable in the COBRA- and polyvalent-vaccinated animals, suggests a predominant role of the humoral response in conferring this breadth of protection (Crevar *et al.* 2015). On the other hand, vaccination of mice with a cocktail of three COBRA H5 VLP-based vaccines, elicited broadly-reactive antibodies recognizing H5N1 viruses from 11 H5N1 clades/subclades isolated over a 12-year span, confirming the versatility and the possibility of further increase the breadth elicited by COBRA immunogens (Crevar *et al.* 2015).

H1N1

As already mentioned, influenza virus infections from the H1N1 subtype have caused pandemic outbreaks in the past.

The most recent, the swine-origin H1N1 pandemic of 2009, reminded the worldwide community of the ever-present menace of a pandemic event. Currently, H1N1 strains are circulating in the human population together with H3N2 strains. Interestingly, it has been reported that in influenza seasons with more H1N1 influenza virus cases, children and younger adults are typically those more affected (Palese 2004).

Since 2009, pandemic-like H1N1 strains are those predominantly circulating nowadays. However, the accumulation of mutations is making them drifting from the original pandemic strain (Van Reeth 2018). In order to overcome possible differences occurring between the vaccine strain and circulating strains, consensus-based approaches have been developed also for the H1N1 subtype. In particular, COBRA H1N1 were designed using sequences of H1N1 viruses spanning the past 100 years, including modern pandemic H1N1 isolates (Carter *et al.* 2016). Each of the COBRA HA antigens was initially expressed on VLP in order to test their immunogenicity and efficacy in a murine model. Interestingly, four of the nine H1N1 COBRA HA proteins had the broadest hemagglutination inhibition (HAI) activity against a panel of seventeen H1N1 viruses, including seasonal and pandemic H1N1 strains. Similarly to COBRA candidates for H5N1, COBRA for H1N1 were used individually, in cocktails or in prime-boost regimens to evaluate the effect on elicited antibodies and protective efficacy compared to H1N1 HA antigens from seasonal and pandemic strains. In this set of experiments, three of the four initial candidates (named P1, X3, and X6) showed the most effective protection in terms of elicited broad HAI response and prevention of infection of mice from a pandemic H1N1 challenge. More in detail, vaccinated mice had little or no detectable viral replication (Carter *et al.* 2016).

Interestingly, these results were also recapitulated when COBRA vaccines were expressed using different formulations, such as live influenza viruses, HA-ferritin nanoparticles and split inactivated vaccines, both in ferret and mouse *in vivo* models (Allen *et al.* 2018, Darricarrere *et al.* 2018, Sautto *et al.* 2018).

Importantly, the COBRA H1N1-elicited antibody response was also evaluated in the context of an influenza pre-immunity (Carter *et al.* 2017). In fact, evaluating the efficacy of candidate broadly-reactive or universal influenza vaccines in a pre-immune setting is of pivotal importance since humans are 'universally' pre-immune to influenza viral antigens due to previous imprinting (Kirchenbaum *et al.* 2017). In this regard, it has been shown that ferrets previously infected with a 1986 H1N1 virus and vaccinated with a single dose of the COBRA HA VLP vaccines, possess antibodies with a broad HAI activity against 11-14 of 15 H1N1 viruses isolated between 1934 and 2013. Moreover, ferrets primed with a COBRA virus infection and subsequently immunized with COBRA VLP vaccines showed a broader HAI activity compared to those subsequently immunized with wild-type HA proteins (Carter *et al.* 2017).

H3N2

Contrarily to influenza seasons in which H1N1 circulating strains are predominant, those in which infections from H3N2 viruses are more frequent, tend to be more severe with a greater number of hospital cases and deaths. This is reflected also in the number of elderly people that are infected with H3N2 viruses, leading to enhanced hospitalizations (Jhung *et al.* 2013).

Using the COBRA approach, a set of candidate H3N2 vaccines were tested in ferrets and mice for their ability to elicit antibodies that neutralize virus infection against not only H3N2 historical vaccine strains but also a set of co-circulating variants that circulated between 2004-2007 (Wong *et al.* 2017). Three of the designed H3N2 COBRA vaccines, expressed on the surface of VLP, were able to elicit antibodies recognizing and neutralizing all the co-circulating strains during this era. Conversely, wild-type vaccine strains were not able to elicit antibodies with HAI activity against these

co-circulating strains (Wong *et al.* 2017). Therefore, the COBRA vaccines have the ability to not only elicit protective antibodies against the dominant vaccine strains but also minor circulating strains that can evolve into dominant circulating strains in the future. Moreover, similarly to previously described *in vivo* pre-immune studies with H1N1 COBRA candidates, ferrets imprinted with historical H3N2 viruses and vaccinated with VLP expressing an H3 COBRA HA antigen (named T10) elicited sera with higher HAI antibody titers than antibodies elicited by vaccines with wild-type HA (Allen *et al.* 2018). Interestingly, sera from pre-immune ferrets subsequently immunized with another candidate H3N2 COBRA vaccine (named T11) even if not endowed with HAI activity, were able to neutralize antigenically distinct H3N2 influenza viruses, suggesting a different mechanism of neutralization such as antibody-dependent cellular cytotoxicity (ADCC) or other antibody Fc-mediated effector functions (Allen *et al.* 2018).

Conclusion

To limit the public health consequences of both seasonal and pandemic influenza virus infections, vaccines that are more broadly and durable protective are needed. Advances in influenza virology, immunology, and vaccinology make the development of a more effective influenza vaccine more feasible than a decade ago. In this regard, the current strategic plan of the US National Institute of Allergy and Infectious Diseases (NIAID) to support the rational design of universal influenza vaccines, aims to precisely characterize influenza immunity and correlates of immune protection. Consensus-based approaches to develop universal or broadly reactive influenza vaccines have shown promising results in eliciting a more effective immune response compared to the current SOC influenza vaccine. However, the mechanism(s) underlying COBRA-elicited antibody breadth remains to be elucidated (Sautto *et al.* 2018). In fact, owing to their design through multi-layer consensus sequence alignments, it can be speculated that COBRA HA immunogens could elicit antibody specificities that recognize conserved epitopes. However, it is also reasonable to hypothesize that their design enables COBRA HA immunogens to overcome the negative attributes of immunodominance and thus elicit an antibody response targeting multiple epitopes. To this end, the dissection of the humoral immune response elicited by COBRA antigens at the single B-cell level is fundamental and would enable the evaluation of their mechanism of elicited breadth of antibody response. Moreover, a molecular characterization of COBRA HA elicited antibodies would yield additional insights into their effector functions endowed by this kind of immunogens.

It is noteworthy that broadly reactive HA antigens for influenza would represent a powerful platform not only from a prophylactic point of view but also for the development of more effective therapeutics (Burioni *et al.* 2009, Burioni *et al.* 2010, Sautto *et al.* 2017). In fact, these novel antigens can be used also for the generation and screening of cross-effective molecules able to recognize and neutralize divergent influenza isolates and to be further developed as possible broad-spectrum antiviral drugs.

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