A Better Understanding of Seasonal Influenza Virus A Evolution to Increase Efficacy of Vaccination for Epidemic Prevention

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Preface

This report is intended as a final thesis for the author's bachelor. Statements, data and conclusions from this report may be used as information provision, but should be handled with caution. This field of research improves at a high rate. Therefore, information and recommendations from this thesis could be outdated in the near future. Always check recommendations with governmental bodies as the CDC.

Abstract

Despite many improvements in surveillance and pharmaceutical development during the past decades, seasonal influenza virus epidemics are not yet under control. The evolutionary characteristics of influenza virus enables escape from immune responses, resulting in yearly epidemics. A better understanding of the antigenic evolution, molecular mechanisms and vaccination strategies have been sought to increase vaccination efficacy and prevention levels against these epidemics. Vaccination against seasonal influenza has become common in most countries globally, but the extent and specific vaccination strategies are not up to date with the knowledge we have today. This thesis explains the mechanisms of the epidemiology of the influenza virus, combined with the molecular knowledge acquired over the past decades and links it to vaccination strategies. Opportunities for better protection and prevention are within our grasp, but it is of importance to put this knowledge into practice and create a better suited strategy to prevent epidemics and pandemics, that populations have been dealing with the past centuries.

Introduction

Annually, 10-15% of the world population gets infected by seasonal influenza virus, with up to a billion infections, and 3 to 5 million severe cases (Clayville, 2011). Approximately, 400.000 deaths are caused by Influenza virus infections every year (Paget, 2019). Vaccination seems a suitable option for epidemic prevention, but efficacy and productivity pose a problem, and vaccines must be updated annually. In the past decades, researchers have sought improvements in vaccination and vaccination strategies by better understanding seasonal influenza virus evolution and vaccination mechanisms.

Influenza virus is part of the Orthomyxoviridae family, characterized by negative-sense, single-stranded and segmented RNA genomes (Bouvier, 2008). Seasonal influenza is divided into three subgroups: A, B and C, but most strains belong to subgroups A and B (Bouvier, 2008). Influenza A viruses originate from swine and birds and adapted to be compatible with humans. Since transmission to humans is possible, they caused multiple pandemics, whereof the Spanish Flu in 1918 and the 2009 Swine flu pandemic are best known (Morens, 2010). Influenza virus A strains can be further categorized based on the combination of haemagglutinin (HA) and neuraminidase (NA) glycoproteins on their surfaces. So far, 18 HA subtypes and 11 NA subtypes are specified (Kosik, 2019). Most subtypes do not occur in humans, but are present in wild birds. In humans, only three combinations have been identified to circulate in the population: A/H1N1, A/H2N2 and A/H3N2. Nowadays, seasonal influenza epidemics consist of A/H1N1 and A/H3N2 subtypes (Morens, 2010). For influenza virus B, no animal reservoir is known, and they are divided into two subtypes: B/Victoria and B/Yamagata, which diverged from each other in the 1970s (Biere, 2010). Influenza virus C is not diverged in subtypes and is not known to have extensive animal reservoirs, and mainly circulates in humans (Petrova, 2018).

As mentioned, influenza virus A is categorized by HA and NA subtypes, which encode for the viral primary glycoproteins. Cell attachment and entry through the sialic acid-binding on the surface of sialylated cells and membrane fusion, is controlled by HA. The cleaving of bonds between HA and sialic acid to

release virions from infected cells is possible through NA (Kosik, 2019). Influenza virus evolves by the gradual accumulation of mutations of nucleotides by the viral RNA dependent RNA polymerase (RdRP) during genome replication (Petrova, 2018). This accumulation and gradual change of nucleotides are called antigenic drift. Another option for antigenic change in influenza virus A is an antigenic shift, which involves recombining HA or NA from different virus strains. The mutation or recombination of amino acids in HA and NA may cause the generation of variants with other characteristics, which leads to the appearance of new antigenic variants. Antigenic changes especially occur in HA, which may result in immune escape from previously acquired immunity. This allows reinfection of earlier protected individuals, and explains the yearly recurrence of influenza virus infections. Consequently, influenza vaccination has to be updated regularly, to keep up with the antigenic changes circulating within the population (Yamayoshi, 2019).

In this thesis, I try to explain seasonal influenza virus A evolution on a molecular basis, and give explanations for the infrequent occurrence of new antigenic variants. Furthermore, I will link transmission and vaccination to explain host-epidemic dynamics and virus-host selection. Consequently, I will give a review of the current vaccination strategies and I will give pointers for improvement of that strategy to prevent future epidemics.

Influenza virus and mutations

Molecular basis of antigenic drift on influenza virus A

The HA protein is the primary target of the human adaptive immune response against influenza virus and the key component used in vaccines (Petrova, 2018). It is synthesized as a single-chain precursor, HA0, during replication and cleaved into the functional globular head (HA1) and stalk domain (HA2) by host proteases (Fig. 1) (Hulse-Post, 2015). Subsequently, a homotrimeric protein forms, where each monomer contains an HA1 and HA2 domain (Wiley, 1981). The majority of antibody-mediated responses to HA are triggered by the highly variable and immunodominant globular head, compared to the more conserved immuno-subdominant stalk domain (Eggink, 2013). In the past decades, researchers focussed on identifying the precise molecular determinants of antigenic drift, and recent studies show antigenic change was mainly caused by single amino acid substitutions at only seven positions in HA immediately adjacent to the receptor-binding site (RBS) in the A/H3N2 virus (positions 145, 155, 156, 158, 159, 189 and 193in H3) during 1968 and 2003. These single amino acid substitutions can be sufficient for the generation of new antigenic variants. Nevertheless, in genetically

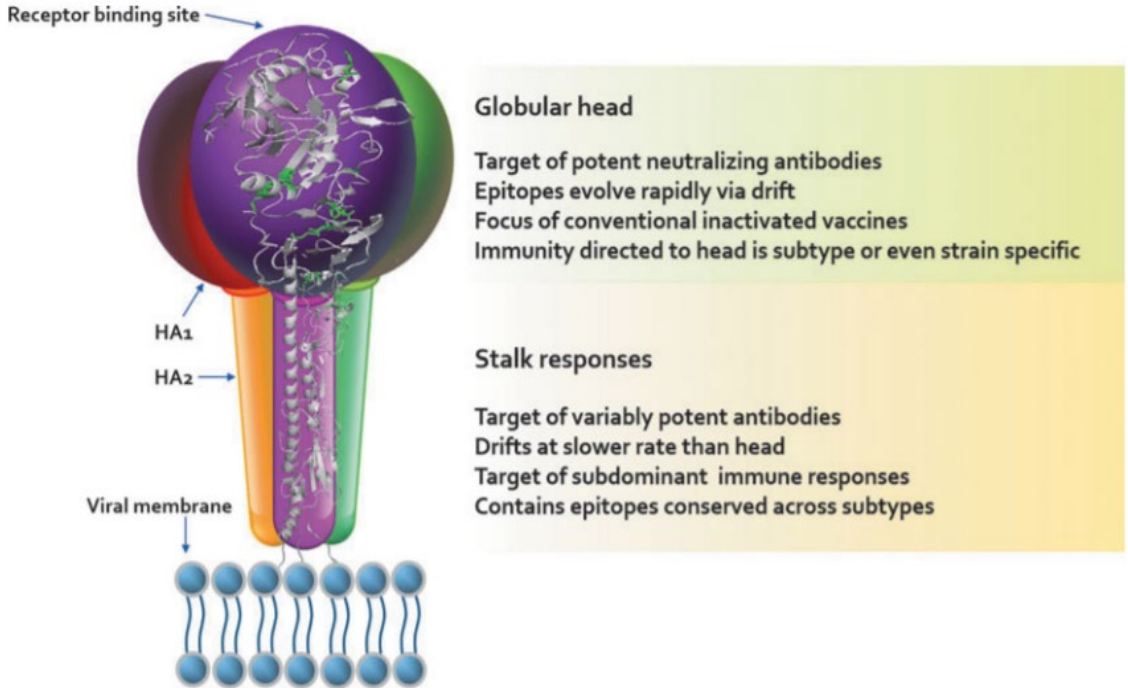

Figure 1. Illustration of HA trimer showing the two major domains, which are the globular head and the stalk. Antigenic and immunologic characteristics are mentioned (Kim, 2018).

different strains, two or three substitutions are required for generation of new antigenic variants. Multiple antigenic variants of the A/H3N2, A/H1N1 (Koel,2013; Klimov, 2012), equine A/H3N8 and avian A/H5N1 strains (Lewis, 2011) were later associated with substitutions on these positions; emphasizing the importance of these substitutions adjacent to the RBS for antigenic evolution. Substitutions in more distant parts from the RBS of HA, associated with fewer antigenic variants, seem to have a more important role in immune escape and cause a more severe disease pattern (Linderman, 2014; Doub, 2017).

Antibodies that target the stalk of the HA protein are less prevalent than antibodies targeting the globular head of the HA. Yet, such antibodies are isolated in higher quantity from older people (Nachbagauer, 2016). Stalk epitopes are less accessible to antibodies than globular head epitopes, limiting exposure to the immune system. Therefore, to obtain the threshold of antibody generation for the stalk protein, multiple infections are required (Nachbagauer, 2016). Given that the stalk region of the HA seems to be conserved among subtypes of the influenza A virus, antibodies against these stalk epitopes may hold potential for inter-subtype protection (Okuno, 1993). Consequently, antigenic escape on the stalk has not been reported widely. Another reason for the rare antigenic escape of the stalk could be the lack of selection pressure because of its inaccessibility to antibodies (Petrova, 2018). Analysis showed that the globular head contains multiple sites where substitution leads to positive selection, meanwhile, the stalk domain only contained one site that positive selection entailed; respectively, H1 hemagglutinin at amino acid position 468 (H1 numbering from methionine) in A/H1N1 and seasonal A/H3N2 (Kirkpatrick, 2018). This site is not located near an RBS and not close to epitopes of cross-reactive anti-talk monoclonal antibodies.

Next to HA, antibodies are generated against influenza virus NA. These antibodies individually, or in absence of HA antibodies, generate protection against disease, reduces viral replication, and disease severity (Marcelin, 2012). However, due to the immunodominance and neutralizing effect of HA, NA antigenic variability has been studied to a lesser extent. NA antigenic evolution is just as HA clustered (Sandbulte, 2011). Nowadays, vaccines do include immunogenic quantities of NA, but the efficacy is not known. The production of vaccines is focused on HA and not on NA, which creates a possibility that NA is compromised during production, since both compounds needs to be treated differently.

Antigenic shift

The second form of antigenic change in influenza viruses happens via antigenic shift. Antigenic drift consists of a gradual change through mutations, where antigenic shift results in a recombination/complete/replacement exchange of HA, and/or NA genes (Webster, 1982). Antigenic shift only occurs in influenza virus A, because its extensive animal reservoirs, which is lacking in influenza virus B. Genetic shift occurs when a reservoir acquires infection of two different strains of influenza virus and a genetic reassortment between strains occurs; facilitated by the segmentation of the influenza virus. Swine seems the best mixing vessels for the emergence of novel recombinant viruses since they possess the sialic α2,3-galactose–linked receptors to which avian influenza viruses preferentially bind , and the sialic acid α2,6-galactose–linked receptors to which human influenza viruses bind preferentially (Wang, 2015). However, the antigenic shift is not limited to swine and can happen in any host infected with multiple influenza virus strains. During the past century, several subtypes occurred. Influenza A virus H1N1, circulated from 1917 until 1957 (Fig. 2) and was replaced by H2N2, which circulated until 1968. Since 1968, H3N2 viruses have been found, and the reoccurrence of H1N1 in 1977 caused the simultaneous existence of two subtypes within the population (Palese, 2002). These antigenic shifts result in limited immunity against these variants, leading to higher morbidity and transmission. It is a rare event, but it is responsible for worldwide influenza pandemics.

Constraints on viral evolution

Viruses are associated with a fast mutation rate and after the understanding that a single substitution is sufficient to generate a new antigenic variant, scientists were surprised that new antigenic variants of A/H1N1 appear every 3-8 years and 3-5 years for A/H3N2 (Fig. 2). Given the fact that hundreds of millions of people are infected every year by seasonal influenza virus epidemics, and every individual will be infected multiple times during their life, it is surprising that new antigenic variants only appear so infrequently.

It seems constraints apply to antigenic drift, and several hypotheses to explain the host-virus interaction and the low occurrence of new variants came up during the past decade. First, it has been argued that a mutation on the diverse globular head, which can cause immune escape, is often

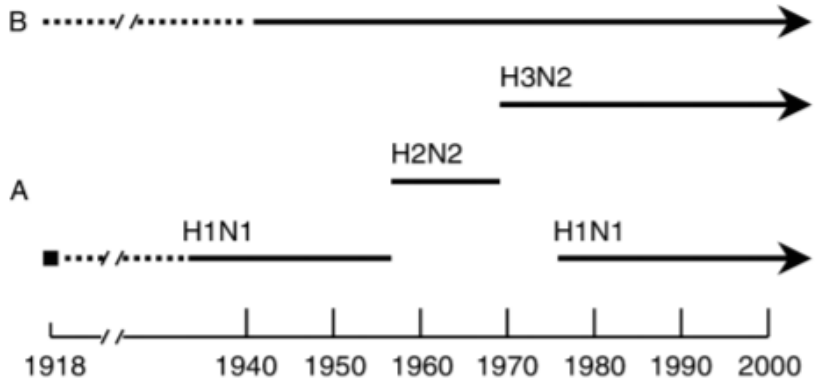

Figure 2.

The epidemiology of human influenza A and B virus. Different influenza A virus subtypes are shown with the time of emerging. Subtypes of influenza virus A consist of a combination of hemagglutinin H1, H2 and H3 and neuraminidase N1 and N2 (Palese, 2002)

accompanied by an impairment of the RBS. Consequently, the virus's ability to enter a host cell is impaired (Bedford, 2015).

Therefore, a mutation that generates immune escape without impairment of host cell entry has to occur, creating a fine balance. This hypothesis could explain an evolutionary event in 2006/2007, where three geographically segregated HA genetic lineages obtained the same amino acid substitution. The same antigenic variant occurred by the K140E substitution. Variants of the A/H3N2 virus showed a similar event (Bedford, 2015). To substantiate this hypothesis, receptor binding avidity for sialic acid could explain these events, since sialic acid-binding is essential for viral entry. Substitutions around the RBS can limit antigenicity, and HA subtypes with lower binding avidity might be less tolerant to substitutions that may influence the RBS in comparison with variants with higher binding for sialic acid.

Secondly, every genetic strain of influenza virus has other characteristics, therefore, other tolerances for different substitutions. It is suggested to be a determinant in antigenic evolution. Overall, the majority of mutations in influenza viruses are lethal or deleterious. The mutation rate of the influenza virus is 2-3 per genome replicated, meaning most replicated genomes contain a lethal mutation (Visher, 2016). Different strains have different tolerances for substitutions, and their ability to tolerate specific mutation varies per strain, but most are lethal, causing high levels of purifying selection and limited genetic variation (Leonard, 2017). Even closely related variants show major differences in toleration of certain substitutions. A theoretical study that used dynamical models of influenza epidemiology and evolution, showed that the rate of antigenic change was influenced and limited because some strains of influenza virus need to acquire multiple substitutions that affect the antigenicity of the protein with little or no impact on the fitness of the virus (Petrova, 2017).

Combining this knowledge, when a substitution that drives antigenic drift occurs, it has still a big chance of not being able to survive and transmit. Because the chance of being accompanied by a lethal of deleterious mutation that limits fitness is present. These mutations offset the benefit that influenza can obtain by single substitutions and the accumulation of these mutations acquired by the virus are a constraint for influenza evolution.

Lastly, even without the constraints, a beneficial substitution has a poor prospect of evading immune selection (which will be explained in selection pressures) and the chance of escape for a variant is nihil. For example, an Influenza A virus RdRP acquires a substitution that could induce an antigenic variant approximately between 2.0×10−6 to ~2.0×10−4 mutations per site per round of genome replication (Parvin, 1986; Nobusawa, 2016). Consequently, a beneficial mutation occurs 2x10^5 per round of replication. Given that an infected cell contains 10^4 virions, the chance of acquiring a beneficial mutation is low, and it is outnumbered by many other virions. After it exits and replication is possible, the virus progeny has to survive the mucociliary system before antibody selection can operate. Conclusively, a small chance of novel antigenic variant survival exists. These hypotheses explain the relatively slow occurrence of new antigenic variants and high within-host competition.

Selection pressures

During prior infections and vaccinations, immunity is obtained, which is the primary evolutionary selection pressures on influenza virus antigenicity. Old antigenic variants go extinct, and new variants emerge, showing an immune selection on the influenza virus (Smith, 2004). However, mechanisms regarding within-host selection poorly understood (Fig. 4a), and research of vaccinated and prior infected people shows minimal effect on the emergence of new antigenic variants.

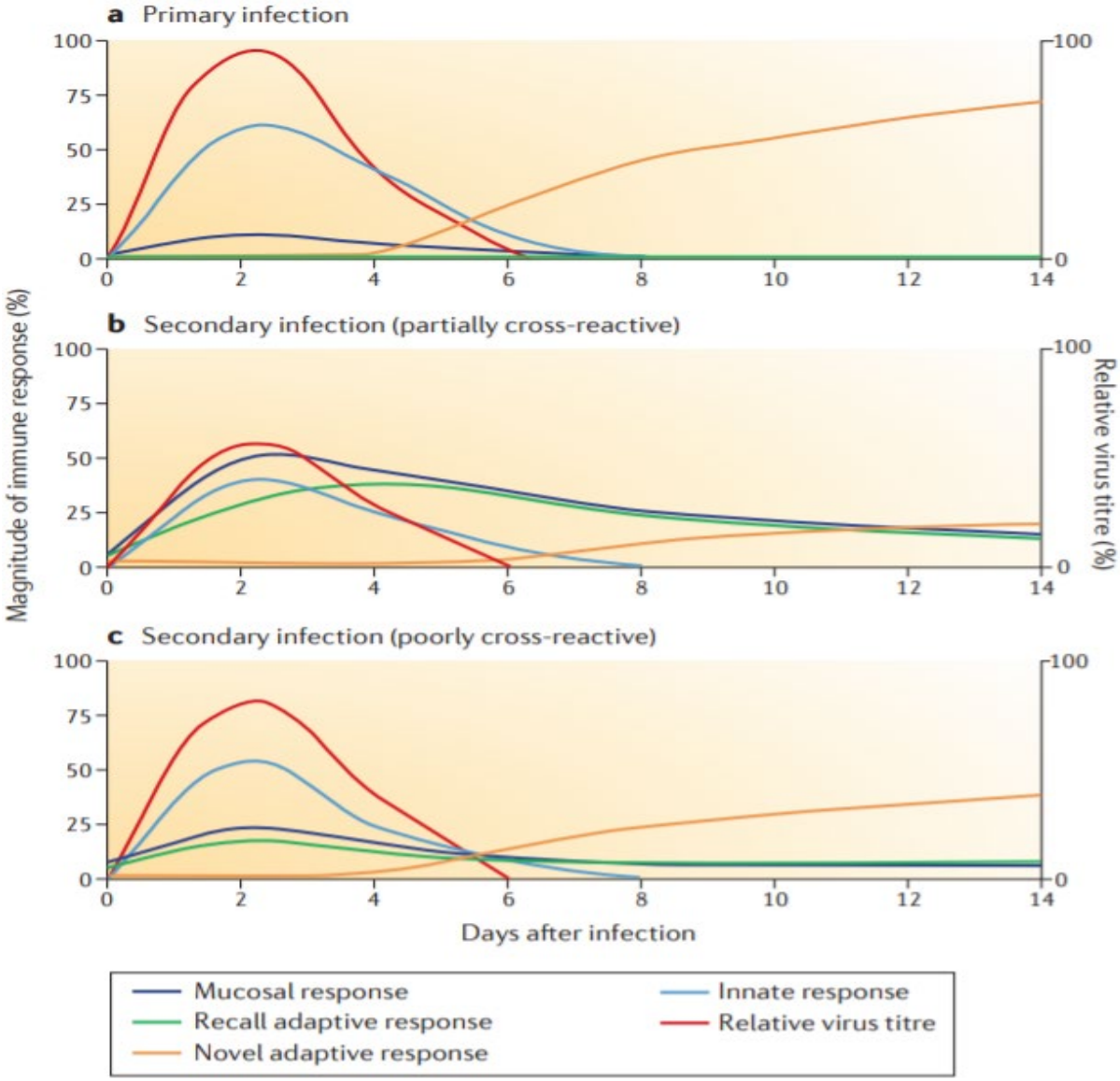

Figure 3. Several models proposed for primary and secondary infection with influenza virus. T y axis depicts the relative contribution of each immune response in correlation with the others. The X axis depicts the time when they become activated. The relative virus titre is shown in as the red line (Petrova, 2018).

1. Shows the immune response of an naive individual

2. Shows the immune response of an secondary infection where immune memory is partially cross-reactive

The roles of innate and adaptive immunity are important to understand within-host selection.

Innate immune selection

In individuals who have not been previously exposed to influenza virus antigens, the first line of defence from the immune system is the innate immune response. The innate immune response is triggered by the recognition of pathogen-associated molecular patterns (PAMPs), which are mainly present on the pathogen or generated during infection. Before receptors are triggered, a mucosal barrier rich in sialic acid, acts as a decoy by binding to viral HA protein. This barrier excludes virus particles from infiltrating host cells, reducing the infectious dose (Wu, 2016). Influenza virus is recognized by several intracellular and extracellular receptors, called pattern recognition receptors (PRR). At least three distinct classes of PRRs can recognize influenza virus, which are: Toll-like receptors TLR3, TLR7 and TLR8, a retinoic acid-inducible gene I (RIG-I) and the NOD-like receptor family member NOD-, NOD-, LRR- and pyrin domain-containing 3 (NLRP3) (Wu, 2011). The PRRs, together with Interferon-mediated responses, result in a rapid establishment of an antiviral state,

limiting the susceptibility of cells nearby infected host cells to replicate the virus. The strength of innate immune reactivity and the efficiency of innate immune selection depends on multiple factors, such as infectious dose, virus immunogenicity that leads to a pro-inflammatory state, and the genetic background. Conclusively, the innate immune response limits the availability of host cells for virus replication, therefore constraining virus evolution and reducing virus diversity. The innate immune response is independent of the antigenic phenotype, causing a fast and first reaction in naive individuals.

Adaptive immune selection

Every year, influenza virus epidemics reoccur and with repeated vaccination, a complex model of immune selection pressures from antibodies and immune memory cells develops. The strength of immune selection varies per individual and depends on several factors, such as previous exposure to virus or vaccine (Fonville, 2014), reactivity or cross-reactivity to an antigen acquired during previous exposure, and the persistence of the presence of memory cells within the host. These factors can influence the immune selection pressures (Slütter, 2017). In individuals without previous exposure, a virus that escapes the innate immune response can replicate freely, which causes virus titres to peak between 24 and 72 hours (Baccam, 2006) (Fig. 3a).

The adaptive immune response creates de novo virus-specific antibodies about 7 to 10 days after infection, causing a low antigenic selection during the primary infection (Fig. 3a). Furthermore, influenza virus infection causes acute and transitory exposure to virus antigens. Therefore, the persistent presence of antigens is needed for a complete and specific generation of adaptive immunity (Ochsenbein, 2000). Consequently, individuals without earlier exposure, acquiring multiple infections or vaccinations are likely needed to generate enough specific antibodies for a highly specific immune response (Valkenburg, 2011). Therefore, even though a high prevalence of infection is present, infections in young children may have a limiting role in virus selection, but might be important as reservoir for novel variants. Secondary infection with an encountered virus induces a mucosal immune response, focusing on the virus epitopes (Fig. 3b). This immune response is mainly mediated by IgA antibodies in the upper respiratory tract, which can cross the epithelial barrier from the blood to the lumen of the respiratory tract to bind and neutralize the influenza virus. Therefore, preventing infection of the host cells (immune exclusion) (Renegar, 2004).

If a previous infection generated specific antibodies, an infection can be rapidly contained, without much additional immune activation. Therefore, after every exposure to a homologous strain, a reduction of serological response occurs (Renegar, 2004). This occurrence affects the selection of antigenic variants strongly and is important during selection pressure for virus evolution within a population. When cross-reactivity against antigenic variants occurs during secondary infection, it leads to incomplete antibody neutralization (Nachbagauer, 2017).

Most antigenic variants of influenza virus have similarities, which causes cross-reactivity, and immune memory after infection. Therefore, after every infection, the recall of previously generated immunological memory causes impairment of the generation of highly specific antibodies for the new variant (original antigenic sin). This occurrence is called antigenic seniority, and individuals typically have the highest cumulative antibody titres against influenza strains encountered in the first years of their life (Fonville, 2014). When secondary infection is acquired, which is partially cross-reactive (Fig. 3b), the immune response will be mainly dominated by immune memory, and a selection pressure against this antigenic variant will occur. However, this is dependent on factors such as: the ability to exist immune responses to neutralize the infection, the acute nature of the infection, and the virus population size on which selection pressure can take place (Kreijtz, 2011).

Acquiring an infection with lower cross-reactivity to previously encountered influenza infections, primarily relies on the innate immune response. It is independent of its antigenic profile, because of the poor response of immune memory and neutralization of existing antibodies. The immune response of poorly cross-reactive antigenic variants is comparable to that of naive individuals and needs a primary generation of specific antibodies before immune memory can be generated. Therefore, virus titres are more comparable with the primary infection (Fig. 3c) (Baccam, 2006).

Between host and epidemic dynamics and spreading

As mentioned, most antigenic variants will be lost before reaching the threshold of replication needed for transmission. Virus population bottlenecks occur during transmission from donor to host and the penetration of innate immune barriers. These bottlenecks substantially decrease the diversity of influenza virus variants (Dylan, 2020) (Fig. 3a).

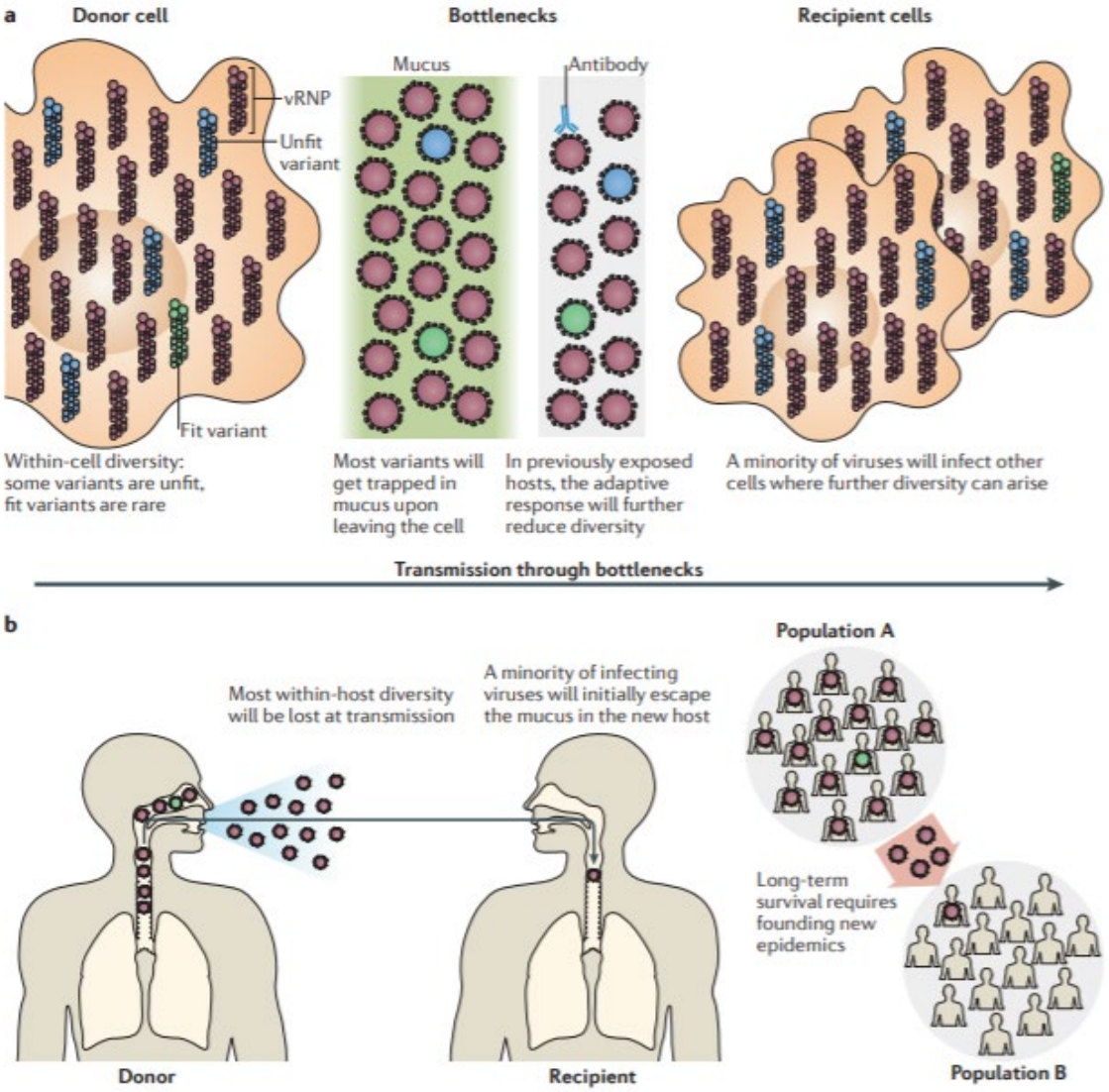

Figure 4. Depicting of the bottlenecks influenza virus encounter (Petrova, 2018).

1. Illustration of within-cell and within-host factors that acts as bottlenecks for viral diversity. Innate a adaptive immunity are depicted.

2. Illustration of the factors between host and population level spread of influenza virus.

The effect of bottlenecks varies, and transmission via respiratory droplets imposes a greater bottleneck than contact transmission (Varble, 2014) (Fig.3b). Larger-scale epidemic dynamics also foist a strong bottleneck, similar to within-host and between individual transmission processes.

New antigenic variants, which are more fit than the dominant variants, have a small chance of survival due to the seasonal nature and short duration of influenza virus epidemics. Therefore, bottlenecks are important for the occurrence of antigenic variants and may have more influence than natural selection on influenza virus variants.

Globally, the timing of seasonal influenza virus varies, with occurrence in temperate regions during the winter and during rainy seasons in tropical regions (El Guerche-Séblain, 2019). Animal models conclude a higher efficiency of respiratory droplet transmission in lower temperatures with lower humidity. Consequently, explaining the seasonal factor of influenza virus epidemics in the temperate parts of the Northern and Southern hemispheres (Lowen, 2007). Nevertheless, in the tropics, influenza epidemics mostly occur during the rainy season, when temperatures and humidity both peak. Showing a switch of primary transmission course and implicates the importance of transmission via contact or fomites, instead of respiratory droplets. Consequently, fomites and contact transmission being the primary causes of viral evolution in the tropics. This observation

would be at odds with data from temperate environment viral evolution. Also, fluctuations in absolute humidity have been linked to the recurrence of epidemics (Peci, 2019). Furthermore, behaviour changes during the winter and rainy seasons are linked to epidemics appearing during those seasons. People tend to increase their time indoors with bigger groups, resulting in an increased chance of viral transmission (Bozic, 2021). In winter, the humidity indoors is lower, facilitating evaporation and keeping initially large droplets suspended in the air as aerosol, increasing the chance of transmission (Bozic, 2021). On the other side, in the tropics, contact and fomites seem to have a more important role in transmission. The periods of higher humidity seem to increase the efficiency of contact virus transmission and the survival of fomites (Lopez, 2013). Lastly, it was found that during winter periods, the immune system has a profound proinflammatory transcriptomic profile. Consequently, proinflammatory responses are upregulated (Dopico, 2015), and may affect the seasonality of influenza epidemics.

The long-term circulation of influenza viruses in the human population is driven via the global movement of viruses. Since the 1800s, epidemic spread has been documented and multiple hypotheses arose, including the movement from North to South when temperatures changes, the persistent presence of viruses, and the migration of viruses from China. Phylogenetic analyses from data sets from the US, South Island, New Zealand, and Australia representing the Northern and Southern hemispheres were conducted (Nelson, 2007). This analysis provided the insight that no persistence of the A/H3N2 virus is present between epidemics, and that they go extinct between epidemics, impugns the hypothesis that the same genetic lineage circulates between regions of the world. Consequently, different genetic lineages circulate between regions and ask for region-specific vaccination strategies.

Differences between global dynamics of influenza virus, result through different rates of virus evolution, influenced by age distributions between regions (Bedford, 2015). To substantiate this hypothesis, regions with lower age distribution have higher rates of virus evolution than older age distributions. The chance of virus evolution is higher within young hosts, since less immune memory is present. Older hosts have a bigger chance of reactive/cross-reactive antibody presence. Therefore, due to less selective pressure in younger hosts, the virus has a longer time to mutate and the onset of epidemics in regions with a lower age distribution is greater. Consequently, older individuals are more important for selective pressure of variants, and the survival of variants in individuals with increased immune memory, gives a greater chance for these variants to onset an epidemic. However, the generation of a new antigenic variant is less likely in a region with an older age distribution. Conclusively, many factors, such as season and age influence the onset of epidemics and vaccination strategies should be adapted per region for best control of influenza virus.

Influenza vaccination

To reduce the burden attributed to seasonal influenza virus epidemics, multiple approaches have been developed over the past decades. Up to now, vaccination is the most important. Ideally, a fully effective vaccine would be available that can prevent influenza infections completely. However, the efficacy of vaccination is limited, and studies show that the current vaccines prevent illness in approximately 70-80% of healthy people under 65 years, and only 30-40% in people older than 65. On the upside, 80% of deaths are prevented by vaccination, even in elderly people (Patriarca, 1985). Currently, three different types of vaccines are licensed, which are inactivated, live attenuated, and recombinant HA vaccines, and each type has its advantages and drawbacks (Yamayoshi, 2019). The efficacy of influenza vaccines relies on the antigenic match to the circulating viruses. Therefore, the seed viruses within the vaccine have to be changed periodically. Currently, twice a year, every six months, the WHO meets to compose vaccination composition. One meeting for the vaccination composition in the Southern hemisphere, and one for the Northern hemisphere. Based on genetic and antigenic characteristics, the most compatible strains to circulating variants are chosen. However, vaccine seed viruses are picked more than six months before injection, which causes an antigenic mismatch between the vaccine seed and the circulating variants.

Current Influenza vaccines

Inactivated vaccines

Inactivated vaccines are produced through vaccine seed virus development in chicken embryonated eggs. Globally, this type of vaccination is most common, due to its low production costs and relative safety. Vaccination with inactivated vaccines is suited when an individual is between 6-12 months old and needs to be retaken annually due to shortly acquired immunity (Young, 2018). Inactivated vaccines are divided into three

subtypes: whole-virion vaccines, split-virion vaccines, and subunit vaccines. Through chemical inactivation with formaldehyde or β-propiolactone, virions in whole-virion vaccines are purified and prepared for vaccination. In the split-virion vaccine, the virus envelope of the whole virion is detached using diethyl ether or detergent treatment. Subunit vaccines consist of HA or NA, which is purified from viral ribonucleoprotein, M1 and the viral envelope (Yamayoshi, 2019). Split-virion and subunit vaccines are the most commonly used inactivated vaccines, despite their lower immunogenicity and narrow range of protection compared to whole-virion vaccines. Threshold titres levels are acquired after many egg passages, especially for the A/H3N2 variant, which may change the antigenicity of HA, influencing the antigenic match (Zost, 2017; Wu, 2017). To prevent this, cultured cell lines can be used for virus propagation instead of eggs, but titres levels are much lower when propagated in cell lines, which results in high costs and low productivity (Hegde,2015).

Live attenuated vaccines

Live attenuated vaccines are used in only a few countries, like the US, Canada and several European countries. They are derived from cold-adapted and temperature-sensitive master donor viruses (Hoffman, 2005) and propagated in eggs. The propagation in eggs simulates a natural infection, without causing major adverse reactions, causing egg-adaptive mutations in HA (Mohn, 2018). The egg stimulates the production of IgA, which occurs as the principal isotype in human immune response in the upper respiratory tract, as well as IgG, which is found in immune responses in the blood, extracellular fluids, and body tissue serum in humans. Therefore, cross-reactive immune responses may occur at the initial replication site (Hoft,2017). The drawback of live attenuated vaccines is that it is not recommended for use in children below 2 years, pregnant women, and people with immune deficiencies, or underlying illness, which is an important target group for vaccination. These groups may induce higher virus titres and experience more side effects (Yamayoshi, 2019).

Recombinant HA vaccines

Recombinant HA vaccines are approved for use by the FDA in the US and use a recombinant-protein-expressing system using insect cells and baculovirus (Richards, 2020). This type of vaccine does not use live influenza virus strains, and mutations can be introduced during the propagation in eggs. A beneficial characteristic of recombinant HA vaccines is the production time of two months, which creates opportunities for epidemic prevention (Richards, 2020). The mechanisms of action are similar to that of inactivated vaccines, however, recombinant HA vaccines require three times the amount of HA the inactivated vaccines require to induce the same amount of antibody titres (Johannson, 2008). Since immune responses are mostly targeted to HA, vaccination must be regularly to match antigenic strains. Also, recombinant HA vaccines are only suitable for use in people between 18 and 49 years, because of their low immunogenicity (Cox, 2008).

Next generation vaccines

Although vaccination is the most common way of protection against seasonal influenza virus epidemics globally, these epidemics have not been controlled. The effectiveness and efficacy of vaccinations must be improved, and advances are categorized in five areas: selection of the vaccine seed virus, targeting the vaccine, use of cultured cells instead of eggs for vaccine virus preparation, increasing the NA content of vaccines, and development of novel classes of adjuvants (Wei, 2020).

Selection of vaccine seed virus

First, next-generation vaccines need improvement of seed virus selection in influenza vaccination. Multiple in silico and in vitro studies have been conducted to achieve this. Epidemics are visualized through the integration of sequence data with epidemiologic information (Klingen, 2017). In silico modelling uses past epidemic data combined with known viral fitness, and molecular knowledge obtained during the past decades. The combining of knowledge enables the creation of a 3D space to predict future possible antigenic directions of influenza (Ito, 2011). To predict which viruses may occur in the future, viruses possessing mutations on the HA head are generated by reverse genetics. Next, variants with characteristics for immune escape are identified via antisera (Li,2016). This type of research enables the determination of antigenicity in future epidemics and may potentially hold a key factor for the identification of future amino acid changes that may occur in the influenza virus. The understanding of molecular changes within strains, and the effect of epistasis has enabled scientist to predict antigenic variant occurrence in a population before they emerge. Next, to value the predictions of variants that will appear within the population at a certain time and create vaccine components is challenging. Antigenically similar variants circulate within human

populations for years, before they acquire a mutation/mutations that lead to antigenic drift (Petrova, 2018). Consequently, a better understanding of triggers for the emergence of antigenic variant creating amino acid changes is needed.

Vaccine targeting

In 2019, approximately 276 million influenza virus vaccinations were distributed globally, meaning only a small part of the recommended people was vaccinated (Sambala, 2019). In perspective, the Center for Disease Control (CDC) advises vaccination for all age groups above six months, except for some exceptions, meaning more than 7,5 billion people globally are recommended to get vaccinated. Young children, the elderly, pregnant women, and people with chronic medical conditions, groups with an increased risk for influenza, are highly recommended to get an influenza vaccination. However, vaccine efficacy differs per group, and there is no differentiation between groups. Antigenic sin becomes a problem for the elderly and the prevention of it during childhood are growing interests for research. In the past decades, much knowledge about the molecular workings and immune activation through vaccination is established, and more vaccination options are existent. Ideally, we should go to a vaccination strategy, where 7,5 billion people are able get vaccinated. At this point, the vaccination capacity is too low and many people are not eligible for vaccination, even though they are willing to. Furthermore, there should be geographically composed vaccines. Up to now, there are 2 different vaccines, for the Northern and Southern hemisphere. This should be expanded to a higher number, where regions with the same antigenic variant circulation get a specific vaccine for those variants. Meaning a well-tailored vaccination strategy for different regions throughout the world. Furthermore, to decrease antigenic sin, different age groups should get different vaccinations.

Improvement of cell-based vaccine productivity

Growing the vaccine seed virus in chicken embryonated eggs is most commonly used, but limitations are present, and with the emerging of the A/H3N2 variant that shows more antigenic changes from egg-adaptive mutations on influenza HA. Therefore, embryonated chicken eggs are not always a viable propagation method anymore (Kishida, 2012; Wu, 2019). Cell-based vaccine production seems a good alternative, but has implications for costs and productivity. Efforts have been made in two distinct areas to increase productivity. Firstly, improvement of vaccine backbone and modification of the virus. To achieve a high virus titre in MDCK, and/or Vero cells and virus modification, several sets of a vaccine backbone are prepared by optimizing the polymerase activity and efficiency of genome packaging, and virion release of the influenza A vaccine viruses (Ping, 2015). Mutations are sought that increases the fidelity of the virus polymerase that is beneficial for genetic stability and vaccine production (Mori,2021). Backbones with such characteristics, combined with HA and NA segments derived from circulating variants, may be utilized as a virus candidate and produced by plasmid-driven reverse genetics (Neuman, 1999). Secondly, the improvement of cells in which virus is produced. This is done by picking clones from parental cells that are very high producing in virus, downregulated in protein expression that suppresses virus growth, and upregulated in human-virus receptor (Rajaram, 2020; Yamayoshi, 2019). Conclusively, these improvements seem promising, and results show an application for future vaccination. More knowledge is needed, but results show that it seems a viable option for precise and relatively quick vaccine production.

NA content

As mentioned, the protection of current inactivated vaccines is primarily mediated by HA, due to its immunodominant characteristics, and HA content in vaccines is measured and standardized. Even though NA has been associated with immunogenic responses and a decrease in viral replication and disease, NA levels are not quantified or regulated. Resulting in a lack of immune response in NA vaccinees (Job, 2018). NA can evoke protective antibodies, which induce neuraminidase inhibition (NI) activity, and give protection via Fc-mediated effector cell activation without NI activity (Yamayoshi, 2019). Antigenic sin and antigenic drift have been reported in NA, but only NA antibodies recognising the epitopes around the enzymatic site inhibiting sialidase activity have been investigated. Therefore, further research about other NA antibodies without the ability to inhibit sialidase activity around the enzymatic site is needed for understanding the importance of NA as an antigen in vaccines. To underline this, NA antibodies, which bind and inhibit N1 through N9 NA activity, have shown to be partially protective against H1N1 and H3N2 virus infections (Job,2018).

Adjuvants

Lastly, novel classes of adjuvants, which are substances that enhance the ability of vaccine

protection via activation of the immune system, hold the potential to improve vaccine efficacy. They create an opportunity for antigens to induce long-term protective immunity. Current adjuvanted vaccines cause predicted side effects, but more frequently than nonadjuvanted vaccines since the adjuvant promotes a stronger reaction from the immune response by mimicking the infection and causing inflammation (Miller,2013). The effect of adjuvants should be monitored since severe adverse effects should be avoided. Novel classes of adjuvants consist of TLR ligands, which are also the best understood. For influenza vaccines, a few adjuvants are added, which are: TLR4 agonists MPLA and GLA, a TLR7/8 agonist, a TLR3 agonist, a TLR9 agonist, and a TLR5 agonist (Anwar, 2019), and seem promising. In combination with the needed cytokine response, and the type I interferon, adjuvants have been incorporated in vaccinations and are being developed (Anwar, 2019). Promising results have been found, but further research is still needed.

Conclusive remarks

In the past decades, we have gained much knowledge about the evolution of influenza virus A, and we come to the point where theoretical and practical knowledge are resulting in ways to combat the recurring seasonal influenza epidemics. However, a major shift needs to be made looking at the current vaccination strategy. By improvements in prediction methods for virus seed composition, creating a more tailored vaccination strategy, invest in propagation methods, research the possibilities of NA and the addition of adjuvants, a great opportunity arises to combat influenza virus epidemics. However, recommendations in this thesis state the most ideal options, and I recognize from a realistic point of view that implementation of all these points are not viable. However, researchers showed that improvement can be made rather easy, but the vaccine industry and governments have not taken enough action to decrease the death toll influenza virus infections take every year.

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