

# Universal Influenza Vaccines

The role of the Haemagglutinin-stem region of influenza viruses in the development of universal influenza vaccines

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**Abstract**

Partly due to the fact that every year new influenza vaccines have to be developed and the inability to develop vaccines against pandemics, scientists started thinking about a vaccine that can protect people against multiple types of influenza viruses. The Haemagglutinin(HA)- stem region might be a possible target for such vaccines. This review article will be about the role of the HA-stem region of influenza viruses in the development of universal influenza vaccines.

The trimeric HA-molecule consists of a globular head, called the H1-domain, and a stem-region, known as the H2-domain. The HA-molecule plays a role in binding of a virus particle to the membrane of a host cell. After binding, the stem-region of HA makes it possible for the virus particle to fuse its membrane with the membrane of the host cell.

In 1992 was the first time that was demonstrated that the HA-stem region might be involved in generating a immune response. After this observation, a lot of antibodies were demonstrated to act against the stem-region. Some studies suggest that a mixture of antibodies might be useful for neutralizing different strains of influenza virus.

Antibodies directed to the HA stem-region prevent fusion of the viral membrane with the endosomal membrane of the host cell. Antibody binding prevents fusion by disturbing the conformational change of the stem-region, just before fusion occurs. The antibody inserts only its heavy chain in a 'pocket' of the stem-region and inhibits the fusion activity.

This review article will provide information about our position in the development of universal influenza vaccines with the HA-stem region as promising target molecule. Also antibodies directed to the stem-region, and their role in developing therapeutic treatment will be discussed, as well as recent discoveries in influenza research, which might be useful in the universal influenza vaccine development.

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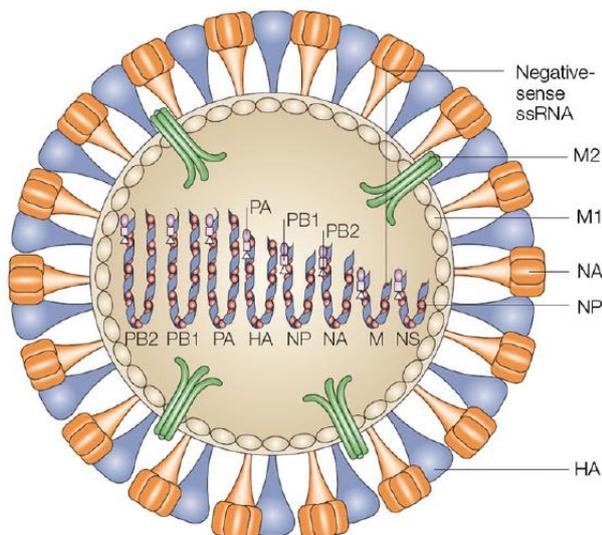
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## Introduction

The development of immunity against influenza virus infections is already seventy years a major topic of scientific studies(1). With the knowledge we have obtained from these studies, it is possible to develop vaccines against influenza viruses. These vaccines can be used to vaccinate people against the influenza virus of the upcoming season. Each year around February, a new influenza vaccine has to be developed, because the virus is changing (2). However, no vaccines can be developed for influenza viruses that cause a pandemic, before this virus has already infected many people as happened with the influenza A/H1N1 virus in 2009. This virus was originally derived from pigs and caused the first pandemic of the 21st century(3, 4). Partly due to the fact that every year new vaccines have to be developed and the inability to develop vaccines against pandemics, scientists started thinking about a vaccine that can protect people against multiple types of influenza viruses. The idea of the 'Universal Influenza Vaccine' was born.

The influenza virus belongs to the family of Orthomyxoviridae.

There are three different types of influenza, which are type A, B and C.



**Fig. 1**

Schematic representation of the influenza A virus. The three surface proteins (HA, NA and M2) are visible on the outside of the particle. Inside the virus particle, the NP and M1 proteins are visible. The virus particle contains eight RNA segments. Two segments (in the figure called HA and NA) encode for the two glycoproteins on the lipid-envelope(5).

The differences between these types can be found in the nucleoproteins (NP) and matrix protein (M1) on the inside of the virus particle (Fig.1) (5). Although all three types can cause illness in humans, only type A is responsible for pandemic outbreaks(4).

The virus particle consists of a lipid-envelope with a negatively charged RNA genome. The influenza A and B types contain eight RNA segments, which encode eleven viral proteins(fig. 1). Each RNA segment encodes for one or two proteins. Two segments encode for the glycoproteins on the lipid-envelope on the outside of the virus particle(5). This lipid- envelope contains two types of glycoproteins (fig. 1), which play an important role in case of infection by the virus. The first important glycoprotein is the trimeric protein 'Haemagglutinin' (HA), which plays a role in the recognition and the attachment of the virus to the host cell(4). Neuraminidase (NA) is an enzyme which is responsible for the destruction of receptors, causing new viruses to be released and makes it possible for viruses to transfer between cells.

Because of the segmented genome, reassortment of the genetic material is possible when co- infection of one host cell with at least two different influenza viruses occurs. Trough point mutations in the RNA genome, the glycoproteins can change quickly, especially HA. This is called the antigenic drift or shift(2). This is the reason that every year new vaccines must be developed(6).

Since the idea of a universal influenza vaccine, three different options have been investigated. The first two options include the development of antibodies against different antigens on the lipid-envelope, the Matrix protein 2 (M2) and the HA-protein. The third option is the development of cytotoxic T-lymphocytes (CTL) directed to the viral proteins NP and M1.

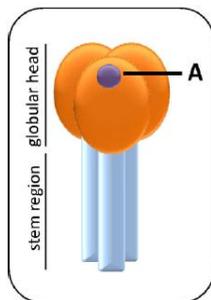
The M2 molecule is partly located inside, and partially located outside of the virus particle (Fig. 1). It functions as a pH-driven proton channel and plays a role in virus replication(4).

Several studies have shown that antibodies against the M2 protein obtained by vaccination, can protect mice against multiple influenza virus subtypes(1).

The reason that one kind of antibody directed to the M2 protein can protect against different influenza strains, is that the M2-proteins, in contrast to HA-proteins, do not change very quickly. That is why viruses with different HA- and/or NA-proteins can still have the same M2-molecules(5). By developing a vaccine that induces the production of antibodies against M2 proteins, this vaccine may provide protection against different influenza virus subtypes.

In 2010 the safety and efficacy of vaccines based on M2-proteins was proven in phase 1 and 2 clinical studies(4, 5).

Furthermore, some research is done to investigate the role of CTLs in the development of a universal influenza vaccine. Several studies have shown that animals infected with influenza virus, develop immunity against multiple subtypes of influenza viruses. This immunity provides a reduction in virus replication, in which CTLs may play an important role(1).



**Fig. 2** Schematic representation of a HA protein with the globular head and stem-region. The head is also referred to as the H1-domain, the stem-region is also known as the H2-region. Because of point mutations in the viral genome, the protein composition in the head of the HA-molecule changes each year(1).

Already in 1992, the HA stem-region antibodies became a subject of interest(7). The HA protein plays an important role in various types of influenza viruses, because it is specific for the kind of influenza virus strain. Like mentioned before, through point mutations in the RNA genome the globular

head can change very easily. The stem-region, however, will often remain unchanged(4). That is why the stem-region is a possible target in the development of universal influenza vaccines.

Several studies have shown that there are different antibodies which act on the stem-region of the HA-proteins(7-9).

It is possible to protect people against influenza virus strains, before they even get infected by the virus. This is called active immunization or prophylactic treatment, and is done by vaccination. Most of the time, people will be injected with inactivated or live-attenuated viruses(2). These virus particles will not cause illness, but the human body will produce antibodies against the antigens which are present on the injected particles. If infection occurs with a living virus particle, the body is prepared and will recognize the virus immediately. Now antibodies will be produced even faster than after vaccination and the virus will be inactivated before it can cause illness. But when a virus has already infected a patient, vaccination does not work anymore. It takes too long before the human body has developed antibodies against the virus particle. In the meantime the living virus has already caused illness and the body will also develop antibodies, but this time to cure the patient and not to prevent against viruses. Sometimes, the human body is not capable of recovering itself. It may need some help what can be done by directly injecting antibodies. This is called passive immunization or therapeutic treatment. The patient does not have to develop antibodies by himself and the virus will be inactivated(10).

This method can be used in patients which are immunocompromised or have a poor immune system, for any kind of reason.

There are some important requirements for a universal influenza vaccine. First of all, the vaccine must be safe and effective, of course. It must be capable of provoking humoral and cellular responses, and in this way provide protection against infection by influenza viruses. Furthermore, it must protect people for longer time and protect against multiple influenza variants.

Eventually, it is also important that the vaccine can be produced quickly and in large amounts, in case a pandemic occurs(2).

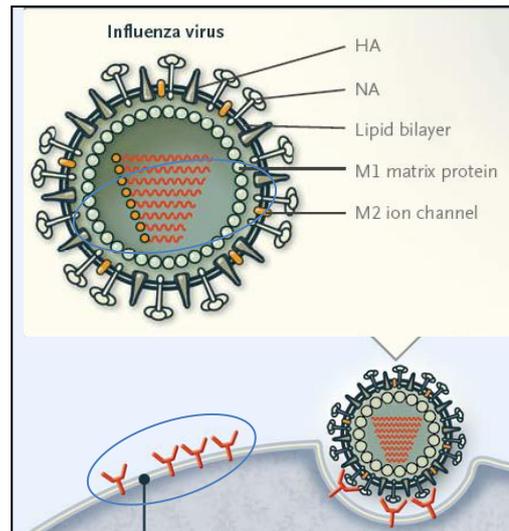
This review article will focus on antibodies directed to the HA-stem region of influenza viruses in the development of universal influenza vaccines.

**Structure and function of the Haemagglutinin-molecule and the stem-region**

HA and NA are the two glycoproteins which are present on the membrane of an influenza virus. As described before, the trimeric HA-molecule consists of a globular head, called the H1-domain, and a stem-region, known as the H2-domain (fig. 2)(6).

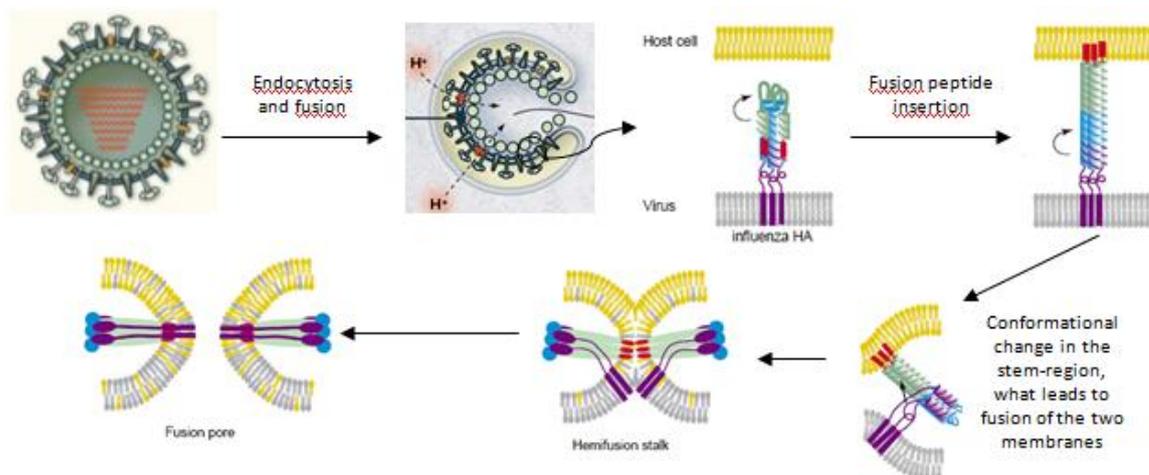
Especially the H1-domain, the receptor-binding unit of the HA-molecule, can change very easily resulting from point mutations in the RNA genome.

There are already 16 different HA subtypes known, called H1 till H16. Together with the 9 different NA subtypes, in theory, all 144 known influenza A strains can be made(4)(6). Based on the structure of the HA-molecule, influenza viruses are classified into two groups; group 1 (containing H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16), and group 2 (containing H3, H4, H7, H10, H14 en H15).



**Fig.3**  
This figure shows an influenza virus particle, together with the membrane of the host cell. On this membrane, the sialic acid receptors (in the blue oval) are shown and endocytosis of the virus particle occurs(2).

There are two main functions of the HA-molecule. The H1-domain of the HA-molecule provides attachment to the host cell by binding with the sialic acid receptors (fig. 3). After this, endocytosis of the virus occurs and because of the low pH, a conformational change in the H2-domain (stem-region) occurs. This conformational change leads to fusion of the viral membrane with the endosomal membrane of the host cell, and the viral genome can enter the cytoplasm of the host cell(fig. 4)(11).

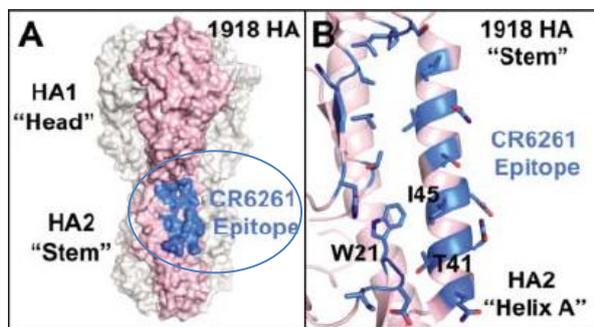


**Fig. 4**  
Schematic representation of the fusion process. The head region of the HA-molecule is not shown in this figure. The fusion peptide inserts in the membrane of the host cell and after the conformational change of the HA- stem region, the two membranes fuse and a fusion pore is generated(2,22).

The reason that the stem-region seems to be a target for inducing cross-reactive immunity, is because it is a highly conserved region in contrast to the head-region of the HA-molecule.

For fusion of the virus particle to the membrane of the host cell, and for productive replication, it is necessary that protease-mediated cleavage occurs between the H1- and H2-domain(6).

The site where this cleavage occurs is simply called the 'cleavage site'.



**Fig. 5**  
Structure of a HA-molecule. In the blue circle, the cleavage site is shown, which plays a role in antibody response to the HA-stem region(21).

This site consists of a loop structure(fig. 5 A and B). The amino acid sequence of this loop (consisting of eleven amino acids) is present in most influenza A viruses subtypes.

In influenza B viruses, the sequence only differs in one or two of the eleven amino acids(6). A study by Fouchier et al. showed that the H2-domain provides a sequence homology of 85% among different subtypes of influenza viruses and a sequence homology of 95% among influenza strains of the same subtype.

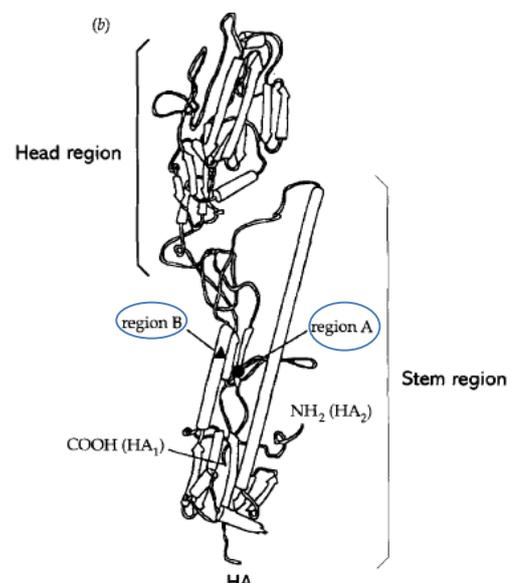
However, other studies show that although there is an antibody response to these amino acid sequences, this response is weak and titers remain low(9, 11, 12).

### The discovery of antibodies against the HA-stem region

In 1992, during a study of Okuno et al, mice were immunized with an H2N2 influenza A strain. When studying, an antibody was found, called C179, which was directed to the HA-

molecule. However, it did not show inhibitory activity to the H1-domain of the HA-molecule, although it neutralized most of the H1 and H2 strains of the influenza A virus(7). There must have been another place on the Haemagglutinin-molecule, where the antibody C179 could bind.

In this study was found that C179 did not prevent attachment of the virus to the host cell, but it prevented fusion of the virus-membrane to the membrane of the host cell. Even when C179 was in a high dilution (1:1.000), the membrane-fusion of both the H1 and H2 influenza strains were inhibited completely(7). But antibody C179 did not inhibit all influenza A strains. Besides that, it was shown that C179 did not inhibit membrane- fusion of H3 influenza strains, and it did not work against influenza B strains. This can be explained by the fact that the stem-region of HA consists of two regions, called region A and region B(fig. 6). The H1 and H2 influenza stains seem to have identical amino acid sequences in the regions A and regions B. The amino acid sequence of the A-region in H3 strains differs only in one amino acid from the H1 and H2 strains, but the B-region differs in five to six amino acids.



**Fig. 6**  
Schematic structure of the HA-molecule with the A- and B-regions of the HA stem-region in the blue ovals. These are responsible for C179 binding to the stem-region in H1 and H2 strains.  
The headless HA in the study of Sagawa et al consists of only the stem-region in this figure(13).

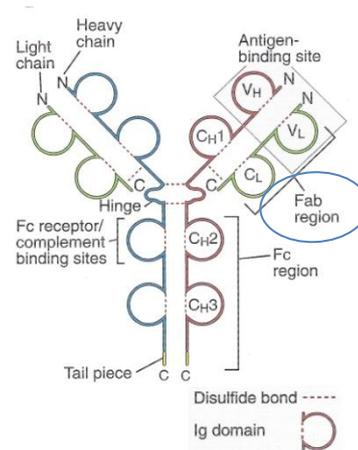
These differences might be the reason that C179 did inhibit the H1 and H2 strains, but had no influence on the H3 strain.

Okuno et al demonstrated that antibody C179 was directed to the middle of the stem-region of the H1 and H2 HA-molecules. At that moment, it was the first time that was demonstrated that the HA-stem-region might play a role in protection against influenza viruses(7).

After this important finding, more studies were done with the same antibody to learn more about the stem-region, its epitopes and about the use of this knowledge in the development of vaccines.

In 1996, mice were immunized with HA-molecules lacking the globular head(fig.6). This mutant, called headless HA, was recognized by the antibody C179, which proved again that this antibody is directed against the stem-region(13). In this study, CV-1 cells (kidney fibroblast cells from African Green Monkey) were transformed with plasmids, which contained a headless HA gene. It was found that mice which were immunized with the transfected CV-1 cells containing the headless HA gene, had a higher survival rate after infection with a mouse-adapted lethal H1N1 influenza A virus, than control mice. These control mice were injected with CV-1 cells containing genes which code for the whole HA-molecule (13). It might be possible, however, that the high C179 antibody response towards the headless HA was found because there was no globular head which can block the stem-region.

Now, almost ten years later, there is a lot more known about antibodies and the stem-region. For example, an IgG- molecule called IgG PN-SIA28 antibody, was investigated for its neutralizing activity. In earlier studies was demonstrated that a Fab fragment called PN-SIA28, recognized epitopes on the HA-stem region and neutralized H1N1 influenza strains. A Fab fragment is the antigen binding site of an antibody molecule (fig.7). It was in earlier studies also shown that the presence of a whole, bivalent IgG-molecule is an essential feature for the neutralizing activity of the antibody.



**Fig. 7**  
Schematic structure of an IgG- antibody molecule. The molecule contains two Fab-regions, which are the antigen binding regions of the molecule(17).

So, in the study of Clementi et al a whole IgG-molecule was generated, containing the PN-SIA28 Fab fragment (14).

The IgG PN-SIA28 antibody was used in tests with human, swine and avian influenza A viruses.

This study showed that the IgG PN-SIA28 antibody had a strong neutralizing activity against most group 1 and group 2 virus strains, including H1N1, H2N2, H5N1 and H9N2. There was, however, no neutralizing activity present for the H7N2 and H3N2 influenza A strains. An important conclusion of this study is, that the epitope on the stem-region is highly conserved(14), as was already shown in the study of Okuno et al(7, 14). Besides the PN-SIA28 antibody, another antibody with the same activity was discovered. This antibody seemed to have the same germ line heavy chain, what might be the reason that the same neutralizing activity was noticed(11, 14).

It was suggested by Ekiert et al, that a combination of antibodies might give a better neutralizing activity than only one antibody. In contrast to IgG PN-SIA28 which did not show neutralizing activity to H3N2 and H7N2, there are antibodies which do have this activity. One of these antibodies is called CR8020(11). This antibody had the same germ line heavy chain as an antibody called CR6261. They have therefore the same neutralizing activity.

These antibodies can neutralize a wide spectrum of H3, H7 and H10 influenza A strains. Their efficacy was demonstrated in mice which were infected with a high, lethal dose of mouse-adapted H3 and H7 virus strains(11).

With the knowledge about the different antibodies, a mixture of two or more antibodies might be effective in a therapeutic treatment for influenza infection. If antibodies directed against group 1 influenza viruses can be mixed with antibodies directed against group 2 influenza viruses a possible therapeutic treatment can be produced.

### The mechanism of antibodies acting against the HA-stem

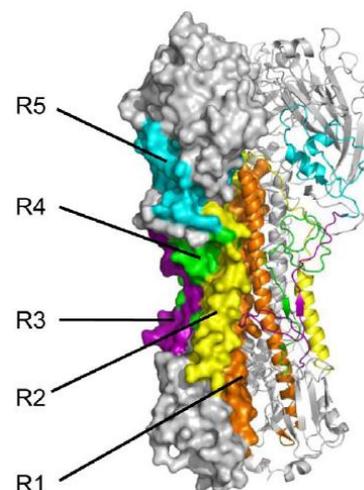
Several studies agree that antibodies directed to the HA stem-region can prevent fusion of the viral membrane with the endosomal membrane of the host cell(7-9, 11).

As mentioned earlier, in 1992 Okuno et al already showed that binding of the antibody C179 to a place on the stem-region, prevents fusion of the virus with the host cell. The consequence is neutralization of the virus(7). How this was possible, was not known at that moment.

Now, there are some possible explanations for this neutralizing activity. It seems that some antibodies, for example CL-385319(15), can stabilize the conformation in the H2-domein at low pH, and destabilize the conformation at neutral pH. Both mechanisms can prevent fusion of the membranes(8). Near the fusion peptide on the stem-region, there is a cavity. This cavity is surrounded by several amino acid residues. Some of these residues seem to play a role in binding of the antibody CL-385319 to the stem-region. There are two critical residues for CL-385319, named N50<sub>2</sub> and F110<sub>2</sub>, which are present in H1, H2 and H5 subtypes. This antibody is shown to be active against different influenza virus strains. These are promising results in the development of universal influenza vaccines(8).

More or less the same results were found in a study, investigating antigenic regions on the HA-molecule. Xu et al found five antigenic regions on the HA-molecule, by expressing virus antigenic peptides on the surface of yeast-cells. With a high-throughput screening method, the relative frequency of each amino acid residue was determined, which were involved in the recognition by antibodies. With the observed frequencies, the position of residues in the 3D-structure of the HA-molecule were determined(16). Four of the antigenic regions were located on the H2-domein, one located on the H1-domein(fig.8). Antibodies which bind these regions, disturb the conformational change of the stem-region, and therefore prevent fusion with the host-cell membrane(16).

In other studies, this is even described in more detail. Sui et al described the crystal structure of the antibody F10, when it is bound at the stem-region of a H5 influenza molecule. They explained, when a conformational change occurs at low pH, this leads to partial burial of the fusion protein. The antibody molecule, consisting of a heavy chain and a light chain, inserts only its heavy chain in a 'pocket' of the stem-region. At that moment, the light chains did not seem to have a function. In this way, the antibody inhibits the fusion activity of the fusion peptide(6, 9, 11).



**Fig. 8** Structure of the HA-molecule, with four antigenic regions (R1-4) on the stem-region of the molecule, and only one antigenic region (R5) on the globular head(16).

### The development of antibodies and the protection of people

There are two ways of helping people when an influenza epidemic or pandemic occurs. The first way is prophylactic, to protect people before they even get infected by a virus. This is especially done by vaccination. The second way is therapeutic treatment. When people are infected by a virus, they can be treated on different ways to neutralize the virus, without producing antibodies by themselves. One possibility is injecting antibodies which are most of the time produced in vitro. Other possibilities are a treatment with an antiviral agent like amantadine, or treatment with a neuraminidase inhibitor like zanamavir(10). The patient does not have to neutralize the viruses by himself, what can induce faster improvement in his illness.

The immune response is a very important mechanism for us to prevent illness. Our immune system consists of innate immunity and adaptive immunity. We are born with our innate immune system and it is the first line of defense against pathogens, for example influenza viruses. When a virus particle enters the body, pattern-recognition receptors (PRRs) recognize the RNA genome. In this case, the viral RNA is called the pathogen-associated marker pattern (PAMP). The PRRs are stimulated and this results in the production of cytokines and type I interferons, IFN- $\alpha$  and IFN- $\beta$ . These interferons play a role in preventing virus replication. Besides that, interferons stimulate antigen presenting cells, like Dendritic Cells (DCs), to present the virus antigens to T cells of the adaptive immune system(1). Macrophages and natural killer cells are also important members of the innate immune system. When macrophages are activated, they can phagocytose cells which are infected with influenza virus and in this way preventing viral spread in the body. NK-cells can lyse infected cells. Activation of NK-cells is based on a balance between signals from activating and inhibitory receptors on the surface of the NK-cell.

When a NK-cell binds a not-infected cell, the activating receptor on the NK-cell binds with a ligand on the surface of the target cell. The inhibitory receptor binds with a class 1 MHC-molecule present on the surface of the target cell and the NK-cell is not activated. The target cell will not be killed. But when the target cell is infected by a virus, the class 1 MHC-molecule will not be expressed and the inhibitory receptor on the NK-cell not bound. Only the activating receptor is stimulated and the target cell will be lysed by the NK-cell(17).

Antibodies play a more important role in the adaptive immune system. When a virus particle is recognized in the body, different cells present antigens on their surface. Many polysaccharide and lipid antigens are able to bind the antigen receptors on B-cell surfaces. B-cells ingest the antigen proteins and degrade them. The peptides will be displayed on MHC-molecules, so helper T-cells can recognize them. These helper T-cells then activate the B-cells, which can produce antibodies against the recognized antigens(17). So it is possible that antibodies can be produced against the stem-region during an infection. Some of these antibodies seem to neutralize not only this influenza subtype, but also bind other subtypes(1, 9, 11).

When a person is vaccinated, inactivated or live attenuated viruses are injected. These particles represent antigens, against which antibodies can be formed. Ledgerwood et al came up with a possibility in vaccine development, based on an immune response against the HA-stem region. They used in their study vaccines which consist of plasmid DNA expressing an H5 influenza HA-sequence. They found an antibody response which was directed to the stem-region. These antibodies seem to neutralize different influenza strains, so this vaccine might be useful in the development of universal influenza vaccines(18). This way of using antigens for vaccination is called gene-based vaccination. Also Wei et al investigated whether gene-based vaccines could elicit neutralizing antibody response.

They also made use of plasmids, but this time plasmid DNA encoding H1N1 or H3N2 influenza HAs were used. Neutralizing antibodies were generated against the H1 influenza strains and were directed to the stem-region of the HA-molecule. It was demonstrated that in non-human primates a antibody response was elicited after vaccination with a HA-encoding plasmid(19).

But when you are already infected with an influenza virus, vaccination will not help you. It will take too long before the human body has developed antibodies against the virus particle. In the meantime the living virus has already caused illness.

In such cases, therapeutic treatment with influenza antibodies might help. This is called passive immunization. The antibodies used for this treatment can be produced by someone who had recovered from an influenza infection(10). But this is not a common way of acquiring antibodies. Most of the time this will be done in vitro. Human B-cells are able to generate antibodies against a specific antigen in vitro, under perfect conditions. However, this is a really expensive and time consuming method (10, 20). When we are able to generate safe and effective antibodies against the HA-stem region in vitro, these antibodies can be a promising therapy for someone who is, for example, not strong enough to generate antibodies by himself.

### Discussion

It may be clear that in ten years of research a lot more is known about the HA stem-region and the mechanism of neutralizing activity of antibodies directed to the stem-region. Now we have to use this knowledge in the development of universal influenza vaccines.

Recent advances in influenza research indicate that the HA stem-region is a very important part of the HA-molecule and might play an important role in vaccine development. Although a lot is known about antibodies generated towards the stem-region, this knowledge cannot immediately be used in the development of vaccines. The knowledge about antibodies is more important for therapeutic treatment.

Ekiert et al came up with the idea of a 'cocktail of antibodies'(11). There have been discovered antibodies which are directed to the stem-region of group 1 influenza strains, there are also antibodies which are directed to the stem-region of group 2 influenza strains. When these two types of antibodies are separately produced in vitro by B-cells, these antibodies can be mixed. The antibody-mix can be used as a treatment for patients which cannot clarify an influenza infection by themselves.

Ledgerwood et al and Wei et al proposed an opportunity in the development of universal influenza vaccines. They investigated the possibilities of a gene-based vaccine. This vaccine contains two (or more) kinds of plasmids, which encode for HA-molecule stem-regions of group 1 influenza viruses and for group 2 influenza strains(18, 19). After vaccination the human body may produce antibodies against these plasmids and will be protected against a large range of influenza viruses.

Of course a lot of research must be done to investigate these possibilities and safety of the vaccine.

Despite all these studies, there are also studies which show that an immune response to the HA2 domain is weak.

Kang et al suggest that a possible explanation would be the presence of the bulky globular head(6). Antibodies might have not enough space to bind the stem-region. However, there are other studies, for example Ekiert et al, which suggest that the conformational change of the HA-molecule which leads to fusion of the membranes, provides enough space for antibodies to bind(11). When the pH is low enough after endocytosis of the virus particle, the conformational change occurs. At that moment the entire head-region is folded and the stem-regions becomes exposed. But this might also be a topic for further research.

A topic for discussion in general, is who ought to be vaccinated. Only the elderly and risk groups, or also people who work in hospitals for example.

Or is it even a good idea to vaccinate everyone, when a universal influenza vaccine is developed? When most people are protected against different influenza types, this saves a lot of costs for the health care in a country. Yet for a normal person who does not belong to a risk group it might be better to not be vaccinated. The immune system has to do the work by itself and this might help the person to get a stronger immune system.

We do not have enough knowledge yet to really develop a universal influenza vaccine. But we become close and I would not be surprised if in the next ten years, universal influenza vaccination will be reality.

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