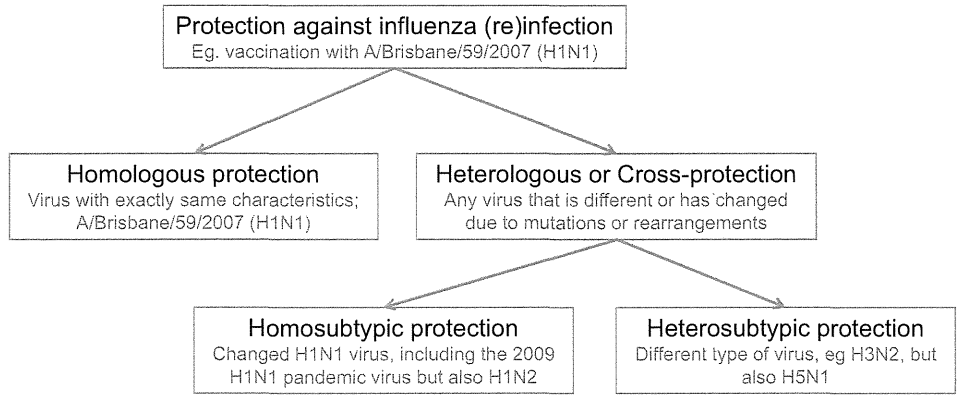

Fig. 1. *Overview of different terms related to different types of protection. Since no official definition is available for these terms, this figure shows how these terms are used* in this review. The definitions are based on the type of haemagglutinin antigen that the virus contains. For each term some virus strains that will fit the term are given in grey, based on an example of protection against A/Brisbane/59/2007 (H1N1).

known to play a role in cross-protection, but in this review we will focus on the current knowledge on the role IgA could play in realising universal protection. Importantly, rational design of IgA inducing vaccines has so far been hampered by a lack of knowledge: since local, tissue-specific, immune responses, including IgA, are often not measured [2], relatively little information is available. Consequently, the importance of the presence of IgA as well as the mechanisms via which IgA responses are induced and maintained are just beginning to be revealed [3].

2. The role of IgA in (cross-)protection from influenza infection

Pre-existing secretory IgA (S-IgA) antibodies can provide immediate immunity via their unique capability to eliminate a pathogen before it even passes the mucosal barrier and enters the human body [4], also termed immune exclusion [5]. Furthermore, IgA has also been shown to be very effective at disarming viruses in virus-infected secretory epithelial cells and in redirecting antigens to the lumen when they enter the lamina propria [3] (Fig. 2). These responses are all non-inflammatory, since IgA, unlike IgG, does not fix complement and thus does not activate the inflammatory complement pathway [6]. Therefore, a strong IgA response could be particularly important in case of highly pathogenic strains, where most complications are caused by uncontrolled pro-inflammatory responses.

Although the roles of S-IgA and serum antibodies are difficult to investigate independently, infection models in knockout mice showed that S-IgA normally does play an important role in protection against influenza [7,8]. Moreover, transfer of S-IgA from respiratory tract washings from immunized to naïve mice was shown to provide protection to challenge with a homologous or drifted strain [9] and several studies in mice showed induction of strong homosubtypic as well as modest heterosubtypic cross-protective IgA antibodies (Table 1).

Thus, IgA contributes to, but is not essential for the establishment of cross-protection to influenza. Interestingly, all mediators of cross-protection, such as CD8 T-cells [27], CD4 T-cells [28] or B-cells [29] seem to be partially redundant, since high degrees of protection were also observed in mice lacking CD4 T-cells, CD8 T-cells or B-cells [21,30,31].

3. IgA production

Antigen specific antibody producing B-cells can develop at two different types of locations, extrafollicular and in germinal centres (GC), and in a T-cell dependent or independent manner [32]. GC function as a specialized environment to support affinity maturation mediated by activation-induced deaminase (AID) induced somatic hypermutation [33]. In addition AID is involved in the production of the preferred antibody class, by influencing class switch recombination (CSR) of the heavy chain [33,34]. Most IgA memory B-cells (B_{Mem}) and long-lived IgA plasma cells develop in the GC of peripheral lymphoid organs and require T-cell help via

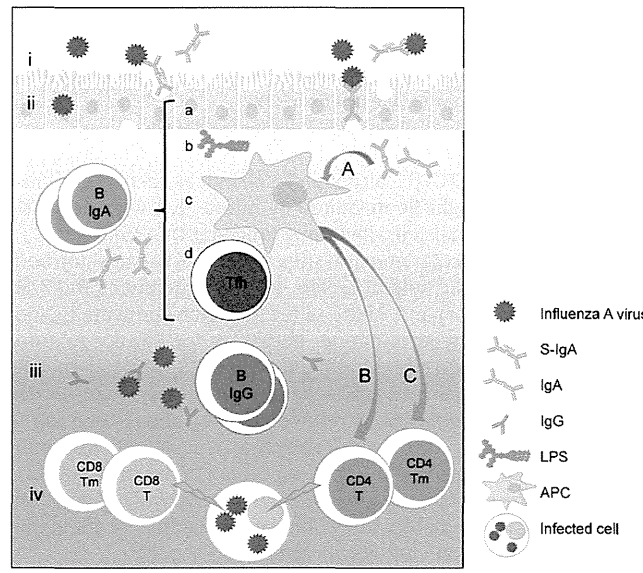

Fig. 2. The role of IgA in protection from influenza infection; function, induction and interaction. Function (i–iv): IgA can neutralize influenza viruses at the mucosal interface (i), even before they actually enter the host by crossing the mucosal barrier, thus existing IgA is the first line of defence upon reinfection or infection after vaccination. In addition, IgA can effectively clear the virus in infected epithelial cells (ii). Virus that could not be destroyed by IgA and successfully entered the host can be eliminated by IgG (iii), which is the main protection on which seasonal vaccination is based, and (iv) cytotoxic CD8 T-cells target host cells that were infected. CD4 cells can also kill infected cells, but are mostly involved in helping B- and CD8 T-cells to eliminate the influenza virus. Induction (a–d): induction of IgA (by influencing for example class switching or proliferation of plasmablasts) is influenced by tissue signals, for example from epithelial cells (a), innate signals, like viral or commensal products such as LPS that activate innate receptors (b), by different types of APC (c) and by helper T-cells, mostly Tfh (d). Interaction (A–C): IgA antibodies were found to influence the function of antigen presenting cells (APC) (A) and so indirectly have a share in the activation of effector T-cells (B) as well as activation of memory responses (C). Tfh: follicular helper T-cell, Tm: memory T-cell, APC: antigen presenting cell, S-IgA: secretory IgA.

Table 1
IgA mediated cross-reactivity to influenza A viruses in a mouse model.

Vaccine type	Adjuvant	Vaccine strain(s)	Cross-responsive strain(s)		Ref.
			Homo-subtypic	Hetero subtypic	
Only homosubtypic					
HA	CTB	H1N1	H1N1		[7]
HA	CTB + 0.2% holotoxin	H1N1	H1N1		[10]
HA	LTB + LT	H1N1	H1N1		[11]
HA	CTB	H1N1, H3N2	H1N1, H3N2		[12]
HA	CTB	H3N2	H3N2		[13]
Homosubtypic and heterosubtypic					
HA	Surf clam microparticles	H1N1 H3N2	H1N1	H1N1	[14]
HA	PolyI:C	H1N1 H3N2	H1N1	H1N1	[15]
HA	Chitin microparticles	H1N1 H3N2	H1N1	H1N1	[16]
HA	Mutant CT	H1N1 H3N2	H1N1	H1N1	[17]
HA	CTB	H1N1 H3N2	H1N1 H3N2	H3N2 H1N1	[9]
HA	CTB	H1N1	H1N1	H3N2	[18]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[19]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[20]
WIV	CT	H1N1	H1N1	H3N2	[21]
Only heterosubtypic					
HA	PolyI:C_{12}U	H1N1, H3N2		H5N1	[22]
HA	PolyI:C_{12}U	H1N1, H3N2		H5N1	[23]
LAIV and IIV[a]	–	H2N2		H5N1	[24]
WIV (formalin)[b]	–	H1N1, H1N2, H2N2, H3N1, H3N2, H5N4, H9N2		H5N1	[25]
WIV (formalin)	+/−LT	H3N2		H5N1	[26]

Only articles are included in which cross-protective IgA antibodies were demonstrated to be present. Vaccines were administered intranasally.
[a] Inactivated influenza virus, different from WIV.
[b] Ether split vaccine did not induce heterosubtypic protection.

CD40L (CD154) and TGFβ1, although T-cell independent B-cell class switching in GC might be mediated by interaction with dendritic cells (DC) and stromal cells, including follicular DC [35].

Also at extrafollicular mucosal sites antibodies can develop both with and without the help of T-cells, the latter involving BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) [36,37]. Although hypermutation, necessary for affinity maturation, was reported to be minimal at these sites [38], antigen-specific antibody producing B-cells that developed at this site were shown to play a role in prevention of reinfection [39], and to lead to the generation of an IgG and IgA producing B_{Mem} subset [40]. Recombinatorial, transcriptional and signalling events underlying IgA class switching were recently reviewed [32]. Below, we will highlight the immunological parameters that could be a target for the induction of IgA production upon influenza vaccination (Fig. 2).

3.1. Mucosal tissue

The inductive sites of the mucosal immune system include mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes. The mechanisms leading to IgA CSR have mostly been studied in the gut and were found to be influenced by the specific environmental factors at this site, mainly created by commensal bacteria and their products [41]. Much less is known about the respiratory tract, which is not populated with as many commensals as the intestinal tract. However, recently it was shown that commensals do play an important role in protection against influenza infection, since antibiotic treatment of mice reduced CD4 T-cell, CD8 T-cell as well as antibody responses, including IgA. Immunity to influenza viruses could be restored by nasal administration of LPS but also by rectal administration of TLR agonists (LPS, CpG, polyI:C) [42]. These findings indicate that signals from distal mucosal regions can support immune priming in the respiratory tract, although it needs further investigation to find whether support from distal regions is preferential over an intact commensal system in the respiratory tract.

3.2. Innate sensing

Influenza viruses interact with several types of pattern recognition receptors, including TLR family members, but also with members of the RIG-I like receptor (RLR) family and the Nod like receptor (NLR) family [43] as well as several members of the C-type lectin receptor family [44]. For vaccination purposes, also members of these receptor families that are not activated by a natural infection have been targeted. For example, flagellin, that activates TLR5, was also shown to promote IgA production and heterosubtypic protection when incorporated in the membrane of influenza VLPs [45]. Similarly, PolyI:PolyC12U, activating TLR3, was shown to induce heterosubtypic protection through IgA antibodies when administered intranasally [23]. Moreover, the effect of this TLR3 ligand was shown to act in a synergistic manner with the TLR-2 ligand zymosan [46].

Recently, several possible models were developed that implicate an essential role for TLR signalling in CSR. Classically, only two signals were described to be necessary to induce CSR in naïve B-cells: presentation of the antigenic peptides on MHC class II molecules after antigen binding to the B-cell receptor and secondly, activation of these B-cells via cytokines and CD40-CD40L interaction with antigen specific T-cells. Currently, TLR signalling

is thought to sometimes provide an important third signal [47] and it has been reported that MyD88 was necessary to induce protection in primary, but not secondary, influenza infection. IgA levels in MyD88$^{-/-}$TRIF$^{-/-}$ mice were reduced in saliva, however, in serum, BALF and nasal wash, levels were similar to those in WT mice and thus induced in a TLR independent manner [48]. In contrast, in another study it was shown that TLRs can play a role in both T-cell dependent and independent IgA responses at both mucosal and systemic levels [49]. This inconsistency might partially be explained by the finding that MyD88 is not only involved in TLR signalling, but it also interacts with TACI (Transmembrane Activator and CAML-Interactor) which is involved in both T-cell dependent as well as independent class switching. Thus, in mice lacking MyD88, both TLR, and TACI signalling will be affected [50].

3.3. APC bridging innate and adaptive immunity

Some antigen presenting cells have been associated with induction of IgA responses, including pDC (plasmacytoid DC), Tip-DC (TNF and Inducible nitric oxygen species (iNOS) Producing DC) and LAPC (late-activator APC).

pDC, highly appreciated in anti-influenza responses for the induction of type 1 interferon, Th1 and cytotoxic responses, were found to also enhance B-cell expansion and differentiation into CD27^{high} plasmablasts upon TLR7 stimulation [51]. Interestingly, pDC were found to be necessary for optimal mucosal IgA and serum IgG production in primary, but not booster influenza vaccination schedules, upon vaccination with live attenuated virus, inactivated whole virus or split virus. In contrast, pDC were not essential for raising a response to live virus [52].

Upon influenza infection, Tip-DC (TNF and inducible nitric oxygen species (iNOS) producing DC) were first known for their production of large amounts of both TNF and NO upon infection with highly virulent strains, thereby inducing tissue damage [53]. However it was recently found that NO – when present in controlled amounts – can induce TGF-βRII expression on B-cells, thereby enabling T-cell dependent IgA class switching. Also, MyD88 signalling downstream of TLR2, 4 and/or 9, needed to induce iNOS, was involved in T-cell independent IgA secretion, in a BAFF and APRIL-dependent manner [54].

Another type of APC that might play an important role was recently identified and designated LAPC, or late-activator APC. Whereas influenza-activated DC are most active around day 3 after infection and induce a response with many Th1 type characteristics, LAPC peak around day 8 after infection and induce a Th2-type polarization, resulting in IgA, IgG1 and IgG2 antibody production, and downregulation of anti-viral Th1-type responses [55].

3.4. Adaptive CD4 T-cell responses

CD4 T-cells are well known for supporting humoral and cellular responses and in addition they can activate innate immunity [28] and display cytolytic potential [56]. The role of CD4 helper T-cells in enhancing B- and CD8 T-cell, immune responses is dependent on the ability of the CD4 T-cells to present antigen on their surface in the context of MHCII molecules as well as the cytokine environment they create.

Importantly, vaccination with peptide variants of the desired epitopes that possess high affinity interactions with the MHC molecules in the host, will increase the amount of these antigens presented on CD4$^+$ T-cells, thereby promoting priming of T and B-cells that interact most efficiently with each other [57]. This could be used to expand the CD4 T-cell repertoire specific for the most genetically conserved regions of influenza HA and NA antigens, thereby enhancing cross-protective neutralizing antibody responses [57]. It was suggested that via priming in the upper respiratory tract, this way also the antigen specificity of IgA antibodies might be influenced by vaccination.

In addition to the MCH molecules, also the cytokine environment created by different T-cell subsets will influence immune responses. The predominant subset of CD4$^+$ T-cells responsible for the generation of high-affinity, class-switched antibodies are follicular helper T-cells (Tfh), that were defined in 2000 by Schaerli et al. as well as Breitfeld et al., based on their surface CXCR5 expression and their key role in antibody production in GC. Tfh are involved in the formation of GC and in the induction of CD40L, IL-2, IL-4, IL-10, TGFβ and IL-21, thereby promoting B-cell proliferation, CSR and somatic hypermutation, resulting in highly specific class switched plasma cells and long-lived memory cells [58,59]. TGFβ was also found to play an important role in IgA class-switching, since mice deficient in TGFβII receptor on B-cells, were hardly producing IgA [60] and S-IgA could not be detected after mucosal vaccination in these mice [61]. Moreover, in the GC, TGFβ and IL-21, produced by Tfh, were found to synergize to stimulate the generation of high numbers of IgA plasmablasts [58].

Recently, it was found that blood circulating cells expressing CXCR5 might be related to Tfh. Three subsets were found; Th1, Th2 and Th17, of which the latter most strongly induced IgA responses [62]. In addition, CXCR5 was also found on a subset of peripheral blood central memory cells and these were proposed to enable quick and efficient secondary antibody mediated immune responses [63].

4. Humoral memory

The essence of vaccination is priming of the immune system with an antigen to induce a quick and effective immune response upon a subsequent encounter of the pathogen bearing that antigen. Success for all current vaccines is based on long-lived antibody production with high affinity, with antibodies shown to be maintained for 75 years after smallpox vaccination [64]. Whether this can be achieved by influenza vaccination needs to be investigated, however, in the serum of people who were naturally infected during the 1918 influenza pandemic, B_{Mem} could be isolated from serum at least up to 70 years after the last encounter [65]. Moreover, these antibodies were also active against the mild A(H1N1)pdm09 [66]. Humoral immune memory is provided by recirculating B_{Mem} and long lived plasma cells, typically residing in the bone marrow [67]. However, much needs to be discovered about the role of B_{Mem} in protection to subsequent influenza infections, regarding for example isotype expression and localization.

In mice, proliferating T-cells were shown to obtain gut- and skin-homing properties during antigen priming in mesenteric and the peripheral lymph nodes, respectively [3]. The factors inducing this are largely unknown, although retinoic acid produced by DC was found to contribute to gut-homing, whereas vitamin D3 metabolites contribute to skin-homing.

It has been stated that probably similar mediators can induce homing to the respiratory tract, with Waldeyer's ring and/or cervical lymph nodes functioning as the antigen stimulation site for T and B-cells with respiratory tract homing properties [3]. Thus, antigens reaching lymph nodes in mucosa-associated lymphoid tissue (MALT) might stimulate mucosal immunity in the same region. Recently it was also found that TGFβ and IL-21, produced by Tfh, not only synergize to induce IgA class switching, but also to simultaneously downregulate CXCR5 and upregulate CCR10 on plasmablasts, enabling their exit from GC and migration towards local mucosa, verifying that Tfh cells play an important role in establishing high-affinity and long-term responses [58]. This was confirmed by a

study that found Tfh to be an important reservoir of memory cells in secondary responses to antigen [68].

B_{Mem} that are produced in GC in the acute phase of infection are known to circulate and to spread to secondary lymphoid tissues. In mice, 8–12 weeks after infection many influenza specific IgA and IgG B_{Mem} were present in these lymphoid tissues, but also in the lung. Upon vaccination, lymph node and lung analysis revealed a higher frequency of IgA B_{Mem} after intranasal compared to intramuscular vaccination, however even upon intranasal vaccination levels of B_{Mem} detected in the lung were very low compared to levels induced by infection [69].

When an intramuscular inactivated vaccine was compared to an intranasal, live attenuated vaccine in a human study, both memory IgG and IgA responses in the circulation were higher after intramuscular administration of the inactivated vaccine, but local memory responses were not determined in this study [70].

Further insight into which homing receptors are involved in B_{Mem} dispersion and how this relates to for example the pathogen, the type of immune activation and the site of induction would be very useful for rational vaccine design. In addition, different types of B_{Mem} might be programmed for specific functions at a specific location. It was recently found that at least two distinct types of IgA B_{Mem} exist: $CD27^+$ and $CD27^-$ IgA B_{Mem}. The former are produced in GC whereas the latter are GC independent and most likely produced locally. The $CD27^-$ B-cells were shown to be induced independent of T-cells, since similar levels of these cells were found in CD40L deficient patients as compared to healthy controls [40]. However, whether these differences are also reflected in their localization and functions, remains to be investigated.

Currently, long-term memory responses induced by influenza vaccines are not tested before use. However, recent studies in mice, ferrets and humans showed that the heterologous protective capacities of prior vaccination depends greatly on the type of vaccine used as well as the type of subsequent vaccination or infection and can be beneficial [71], but also detrimental [72–74]. The latter could have severe consequences in case of a pandemic and was therefore intensively studied using data based on A(H1N1)pdm09 infections. However, conflicting data have been reported and prior vaccination with a seasonal trivalent vaccine has been associated with either increased illness due to A(H1N1)pdm09 [75,76], no association [77–79] or an association with protection of A(H1N1)pdm09 related illness [80–83]. Importantly, all these studies represent association studies and many confounding factors might be unknown, as is stated by the authors as well. Thus no thorough data are available, but improved epidemical studies will pose a great challenge since it is extremely difficult to take the full history of exposure to influenza antigens in humans into account.

Using a different approach, research on human monoclonal antibodies derived from plasma cells or B_{Mem} suggested that vaccination for seasonal influenza would mainly lead to activation of B_{Mem} responsive against dominant epitopes. In contrast, exposure to an antigen representing a major antigenic shift would increase chances of activating and expanding rare heterosubtypic B_{Mem} recognizing highly conserved epitopes, due to absence of competition by pre-existing B_{Mem} recognizing the dominant epitopes [84,85]. Thus, subsequent vaccination with several highly diverse influenza variants might induce good cross-protection. Importantly, the induction of long-term responses should also be studied, since it was shown that only antibodies derived from B_{Mem}, but not from long living plasma cells, were able to strongly neutralize escape mutants from West Nile virus [86].

5. Implications for vaccine design

Seasonal influenza vaccines are currently prepared, based on the prediction of the strain that might cause the epidemic in the following season. These vaccines are mostly injected intramuscularly or subcutaneously and are designed to prevent the onset of the disease induced by the specific vaccine strains, but these vaccines neither induce cross-protection nor prevent infection, since they mostly induce neutralizing IgG antibody in the serum (Fig. 2).

In contrast, influenza vaccines that are currently being designed are mostly based on other mechanisms than induction of IgG, that induce a broader cross-protection, with the ultimate ambition a so called 'universal influenza vaccine'. Several characteristics of the influenza virus as well as the people that should receive the vaccination have to be taken into account. The diverse and more cross-responsive response upon infection compared to current vaccination would suggest that the induction of several, if not all, immunological effectors that can add to cross-protection would more likely be capable to optimally kill viruses with diverse characteristics and at different locations in the body (Fig. 2). Reasoning based on the vaccine recipients would lead to the same conclusion, since people of different ages, sexes as well as genetic and environmental backgrounds will respond differently to vaccination and might rely on different immune mediators for their protection [28,87]. This is in agreement with prospective findings on the key immunological responses induced by the successful yellow fever vaccine, which was identified as 'broad, polyfunctional and persistent, integrating all effector cells of the immune system' [88]. Importantly, different effector cells will not only work next to each other, but will also interact. For example, CD4 T-cells facilitate B-cell responses but B-cells were reported to in their turn also influence CD4 T-cells in several ways [89]. Also IgA was shown to influence T-cells, since $IgA^{-/-}$ mice showed reduced T-cell priming and memory responses upon influenza vaccination, due to impaired APC function that could be overcome with IL-12 [90] (Fig. 2). More knowledge on these interactions will further take vaccine design out of its traditional methodology based on trial-and-error, towards a more rational approach.

Importantly in this respect, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated viruses within a particular subtype of the A-type virus in humans [91–93]. Inactivated virus in its turn has been shown to be more immunogenic than split vaccines, which is in agreement with the general finding that effectiveness and safety of vaccines are usually inversely correlated.

Thus, both whole virus particles as well as split-product seasonal vaccines can induce strong protection against the homologous virus [94]. However, heterosubtypic immunity is not observed when vaccination is performed using an ether-split vaccine, whereas in the same study administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity [25]. The stronger immunogenicity of the inactivated whole virion vaccine in mice was likely due to the stimulation of innate immunity by genomic single stranded RNA, via TLR7 [95,96]. Since most viruses produce dsRNA during replication [97], synthetic dsRNA can likely act as a partial molecular mimic of viral infection.

This has been confirmed in a study where intranasal administration of an ether-split vaccine from PR8 (a H1N1 type influenza strain) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine which are antigenically different from A/PR8, in the presence of poly(I:C) conferred complete protection against A/PR8 virus challenge in a mouse model of nasal infection, suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses [15]. Clinically safe when administered intravenously, with intranasal administration currently in the pre-clinical phase and recently shown to be a potent

inducer of innate immune responses upon subcutaneous administration [98], dsRNA, poly(I:C_{12}U)(Ampligen), was investigated as a dsRNA adjuvant for intranasal avian influenza vaccines [22].

The stronger immunogenicity of the live virus compared to the whole inactivated vaccine may be caused by many mechanisms other than stimulation of TLR7 or 3, such as additional receptors involved or a different biodistribution or kinetic profile of live virus compared to inactivated vaccines. The former might be mimicked by using a ligand for those receptors as an adjuvant, the latter two might possibly be mimicked by the use of different carriers for the antigens that will influence kinetics as well as biodistribution [99].

While we are currently still learning from influenza virus infections, ultimately we would like to design vaccines that outclass natural infections. This might be achieved by careful selection of highly conservative parts of influenza membrane proteins, in combination with several adjuvants that together will activate the required broad spectrum of tissues and cells.

A very promising combination might be nanoparticles, mostly associated with enhanced CD8+ T-cell responses, and TLR ligands, that together can induce very strong and broad humoral responses via induction of GC formation and expansion of Tfh cells [100]. For the rational design of effective vaccines directed against different pathogens, increased understanding of the mechanisms of single as well as combinations of adjuvants in great detail [98,100] will be indispensable.

Notably, recent clinical trials revealed that the intranasal administration of a whole inactivated influenza virus to healthy human subjects, without adjuvant but with a prime-boost regimen, induced high levels of nasal neutralizing antibodies that consisted primarily of polymeric IgA (unpublished data). Whether the absence of adjuvant was less important in human subjects because of the pre-existence of (cross-)protective memory due to a history of infections and/or vaccinations, we are currently investigating.

In conclusion, the induction of IgA will broaden the immune response induced by vaccines, by introducing local immune responses, adding to cross-protection, balancing pro-inflammatory responses and making memory similarly more diverse (Fig. 2). That IgA alone will most probably not be able to induce full protection in case of a heterosubtypic infection could actually be an advantage, since partial protection by IgA will reduce the viral load, while leaving enough space for the cellular immune system to get primed. This way, innate, humoral and cellular responses will all be activated, resulting in the strongest renewal of the immunological memory and ensuring the best possible preparedness for the next influenza virus that will be encountered.

Acknowledgements

E. van Riet is a recipient of a Postdoctoral Fellowship for Foreign Researchers of the Japan Society for the Promotion of Science. This study was further supported by grants from the Ministry of Health, Labour and Welfare, "H19-Trans-Ippan-002" and "H23-Shinkou-Ippan-015". Also, we would like to acknowledge all scientists whose work could not be cited due to restrictions in the number of references.

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Journal of Medical Virology 84:336–344 (2012)

Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

Grant sponsor: Japanese Ministry of Health, Labor, and Welfare.

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Received 3 October 2011
DOI 10.1002/jmv.22273
Published online in Wiley Online Library (wileyonlinelibrary.com).

and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the

nasal cavity several times using a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) filled with saline solution according to the manufacturer's instructions. Pieces of dental cotton (Dental Cotton Roll; B.S.A. Sakurai, Nagoya, Japan) were then immersed in the collected nasal washes. Dental cotton pieces (containing a combined absorbed volume of about 25 ml of nasal wash) were then placed into a filter insert (Oxi Fil filter insert; TOHO, Tokyo, Japan) with bottoms drilled to create several pores, and placed in 50 ml polypropylene centrifuge tubes. Clean nasal wash was separated from mucopolysaccharides and other debris by centrifugation at 2,200*g* for 5 min at room temperature. This procedure was repeated for the entire 100 ml nasal wash sample from each participant. The pooled, clean nasal wash was then concentrated to a final volume of approximately 0.5 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash was stored at −80°C before use.

Quantitation of IgA, IgG and IgM Antibodies and Other Proteins

The levels of human IgA, IgG, and IgM antibodies in the nasal wash and serum samples were estimated using human IgA, IgG, or IgM ELISA kits (Bethyl Laboratories, Montgomery, USA). The level of human serum albumin in the nasal wash samples was estimated using a Human Albumin ELISA kit (Bethyl Laboratories). The protein concentration in the samples was measured using either a BCA Protein Assay Kit, or a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization Assays

The level of serum antibodies against the vaccine viruses was examined using micro-neutralization assays as previously described [Belshe et al., 2000; Kadowaki et al., 2000] with minor modifications. In brief, serum samples were treated with a receptor-destroying enzyme (RDE(II); Denka Seiken, Tokyo, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C before use. The first dilution tested in the assays was 1:10. The concentrated nasal wash samples [1 mg/ml total protein, corresponding to about 1/10 of the total IgA found in nasal mucus (2.20 mg/ml)] [Kurono and Mogi, 1987] were also treated with RDE(II) and heat-inactivated before use. The first dilution tested in the nasal wash assays was 1:20. Twofold serial dilutions of the serum samples were mixed with an equal volume (50 μl) of diluent containing influenza virus equivalent to 100 $TCID_{50}$. Each mixture was added to the wells of a 96-well plate containing a monolayer of MDCK cells. Four control wells were included on each plate and contained either virus or diluent alone. The plates were then incubated for 4 days at 37°C in a 5% CO_2-humidified atmosphere. The monolayer in each well was observed for the presence or absence of cytopathic effects, fixed with 10% formalin for more than 5 min at room temperature, and stained with Naphthol blue black. After the plates were washed and dried, the stained cells were solubilized with 0.1 M NaOH and the absorbance (A) was measured at 630 nm. The average $A_{630\ nm}$ value was determined from quadruplicate virus-infected wells (A_{virus}) and cell culture-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula: $X = (1/2) \times (A_{cell} - A_{virus}) + A_{virus}$, were considered positive for neutralization. The titres recorded were the reciprocal of the highest dilution, where A_{630} was >X.

Hemagglutination Inhibition

The antibody responses to the vaccine viruses were examined in serum and nasal washes using HI antibody assays incorporating a microtiter method as described elsewhere [Hierholzer et al., 1969]. All samples were pre-treated with RDE(II) at 37°C for 18 hr, subsequently inactivated at 56°C for 30 min, and mixed with packed red blood cells to remove any nonspecific inhibitors. The starting material for the assays was a 1:10 dilution for the serum samples and a 1:40 dilution for the nasal wash samples.

Fractionation of Nasal and Serum Samples

The concentrated nasal wash samples (100 μl, 6 mg/ml) and diluted serum samples (10-fold dilution, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 10/300 GL gel filtration column using an FPLC-AKTA chromatography system (GE Healthcare, Little Chalfont, UK). The concentrated nasal wash sample was treated with 1 μg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C to decrease the viscosity and then centrifuged using Vivaspin to remove the lysozyme prior to gel filtration. Fractions (each 500 μl) were collected in PBS at a flow rate of 0.1 ml/min; little or no change in the fractionation pattern of the antibodies in the concentrated nasal wash samples was observed following lysozyme treatment. Molecular weight marker proteins (Kit for Molecular Weights 29,000–700,000 Da; Sigma-Aldrich) were eluted under the same conditions to determine the size of each fraction.

RESULTS

Measurement of Neutralization and HI Titres in Concentrated Nasal Wash Samples

The total protein level and the levels of IgA, IgG, and IgM and human serum albumin in 100 ml of unconcentrated nasal wash and in approximately 0.5 ml of concentrated nasal wash are shown in Table I. About 70% of the total nasal wash proteins were lost during the concentration process. Also, a fraction of the higher molecular weight (MW) proteins and lower MW proteins (less than 30 kDa) was lost by

TABLE I. Concentration of IgA, IgG, IgM and HSA in 0.5 ml of Solution Concentrated From 100 ml of Nasal Wash (n = 10)*

Unit	Concentration: Mean ± SD				
	Total protein	IgA	IgG	IgM	HSA
Nasal wash (n = 10)					
Unconcentrated					
mg/100 ml	5.875 ± 1.856	1.132 ± 0.678	0.125 ± 0.057	0.032 ± 0.021	0.531 ± 0.280
Concentrated					
mg/0.43 ± 0.06 ml	1.647 ± 0.549	0.375 ± 0.193	0.093 ± 0.044	0.007 ± 0.006	0.292 ± 0.214
Concentration calculated in terms of total protein (mg/ml)	1.00	0.217	0.057	0.004	0.177

*The concentration was calculated using two nasal wash samples collected from five participants (with a 1 week interval).

adsorption to the cotton and during Vivaspin centrifugation, respectively. However, better recovery was observed for IgA and IgG. When the concentration of the enriched nasal washes was adjusted to 1 mg/ml total protein, the amount of IgA was 0.217 mg/ml. This amount of IgA in the concentrated nasal wash corresponded to about 1/10 of the levels of total IgA recovered from nasal mucus (2.20 mg/ml) by aspiration as reported by Kurono and Mogi [1987] (Table I). In subsequent experiments, neutralization and HI titres in the nasal wash samples were measured using concentrated nasal wash proteins (1 mg/ml of total protein), which contained 1/10 of the IgA found in mucus, to ensure that the nasal and serum neutralization titres were assayed at equivalent levels.

The amount of total IgA and total IgG in the nasal wash samples from each participant varied slightly at each sampling time. Also, the level of total IgA and IgG antibodies did not increase significantly between pre-vaccination and post-vaccination in any of the participants. Thus, the average amount of total IgA or total IgG in the nasal wash samples from the five participants was relatively constant (data not shown).

Neutralizing Antibody Responses in Nasal Wash and Serum Samples

Next, antibody responses in the nasal wash and serum samples were examined in all five study participants. The responses are presented as neutralization titres against the A/Uruguay (H3N2) virus in Table II. The responses recorded in the four young adults (between 18- and 50-years-old) are also shown as geometric neutralization titres (Fig. 1). The nasal wash and serum neutralization titres increased in all participants as the number of vaccinations increased, although the degree of increase differed between participants. In addition, nasal wash neutralization titres increased more rapidly than serum titres. The nasal wash titres showed at least a fourfold increase after the second vaccination in the four young participants (all of whom had a nasal wash neutralization titre of 1:20 or 1:40 before vaccination). By contrast, a fourfold increase in the serum titre was observed only after the fifth vaccination in three of the participants (all of whom had serum titres of <1:10, 1:20, or 1:40 before vaccination). Participant P5, who was 67 years old, showed at least a fourfold increase in nasal wash titre after the fourth vaccination, but no significant

TABLE II. Neutralizing Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

	Neutralization titre against A/Uruguay virus (H3N2)[a]									
	P1		P2		P3		P4		P5	
Weeks (vaccination)	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	20 (1)	40 (2)	20 (1)	<10 (<0)	40 (2)	20 (1)	20 (1)	<10 (<0)	20 (1)	<10 (<0)
3 (2nd)	80 (3)	160 (4)	20 (1)	<10 (<0)	80 (3)	20 (1)	20 (1)	<10 (<0)	40 (2)	<10 (<0)
6 (3rd)	160 (4)	160 (4)	80 (3)	10 (0)	320 (5)	20 (1)	80 (3)	<10 (<0)	40 (2)	<10 (<0)
9 (4th)	320 (5)	160 (4)	160 (4)	20 (1)	1280 (7)	40 (2)	160 (4)	10 (0)	40 (2)	<10 (<0)
12 (5th)	320 (5)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	80 (3)	10 (0)	80 (3)	<10 (<0)
15	1280 (7)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	160 (4)	20 (1)	160 (4)	10 (0)
28	640 (6)	160 (4)	160 (4)	40 (2)	1280 (7)	80 (3)	N.D.	N.D.	80 (3)	10 (0)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

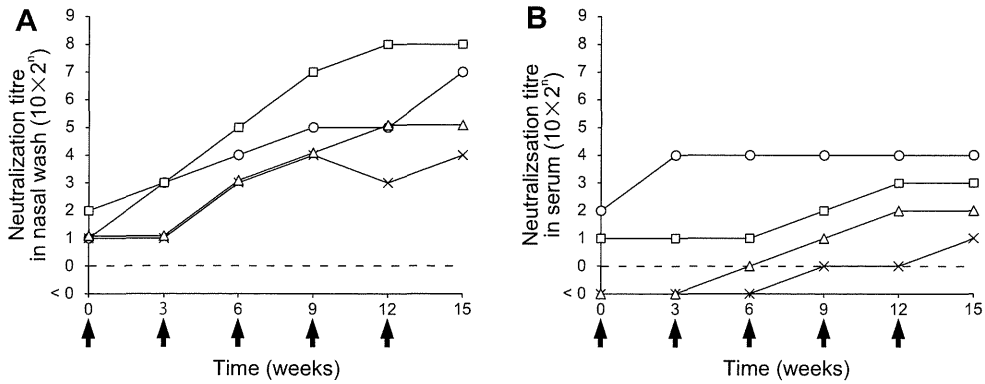

Fig. 1. Nasal wash and serum sample neutralization titres against A/Uruguay (H3N2) (pre- and post-intranasal immunization). Neutralization titres against the A/Uruguay virus in nasal washes (**A**) and serum (**B**) were determined in samples collected from four participants (18–60 years old; P1, open circle; P2, open triangle; P3, open square; and P4, cross). The participants were given five doses of the threefold concentrated A/Uruguay split influenza vaccine intranasally with an interval of 3 weeks between doses (each arrow indicates a point of vaccination). The neutralization titre shown is the geometric titre.

increase was observed in serum titre after five vaccinations. In all participants, the nasal wash and serum titres were largely maintained, even at 16 weeks after the fifth vaccination, at which point the nasal wash titre decreased only slightly, while no decrease was observed in the serum titre (Table II).

HI Antibody Responses in Nasal Wash and Serum Samples

Antibody responses were also examined by measuring the HI titre against the A/Uruguay (H3N2) virus. Table III shows the pre-vaccination HI titres of the nasal wash and serum samples from two participants, and the HI titres 3 weeks after each of the five vaccinations. For each participant, the HI titres were lower than the neutralization titres shown in Table II. The HI titres were approximately 1/4–1/8 the level of the neutralization titres. Statistical correlation analysis of the data presented in Tables II and III showed a strong correlation between the HI titres and the neutralization titres ($r = 0.8699$). Thus, the HI titre correlated with the neutralization titre, although it was less sensitive than the neutralization titre.

TABLE III. Hemagglutinin Inhibition (HI) Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

	HI titre against A/Uruguay virus (H3N2)[a]			
	P1		P2	
Weeks (vaccination)	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	N.D.	10 (0)	<40 (<2)	<10 (<0)
3 (2nd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
6 (3rd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
9 (4th)	40 (2)	20 (1)	<40 (<2)	10 (0)
12 (5th)	40 (2)	20 (1)	80 (3)	20 (1)
15	160 (4)	40 (2)	80 (3)	20 (1)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

Fractionation of The Nasal Wash and Serum Samples

The types of antibody present in the nasal wash and serum samples were examined after fractionation on a gel filtration column. The concentrated nasal wash samples (100 μl, about 6 mg/ml) and diluted serum samples (10-fold diluted sera, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 column in PBS. The antibody concentration in each fraction was then measured by ELISA. Figure 2 shows the profiles for IgM, IgA, and IgG antibodies, together with the absorbance values for the total protein in each fraction. The nasal wash samples contained IgM, which comprised less than 1% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 20% of the total protein and showed a peak MW of about 660 kD; and IgG, which comprised about 6% of the total protein and showed a peak MW of 150 kD. The MW of the nasal IgA (150 kD–900 kD, with a peak MW of 660 kD) appeared to correspond to that of tetrameric IgA (the MW of dimeric IgA is estimated to be about 360 kD). The maximum absorbance value observed in the protein profile (at around 66 kD) was due to the presence of human serum albumin (Fig. 2A).

The serum samples contained IgM, which comprised about 3% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 6% of the total protein and showed a peak MW of about 150 kD; and IgG, which comprised about 23% of the total protein and showed a peak MW of 150 kD

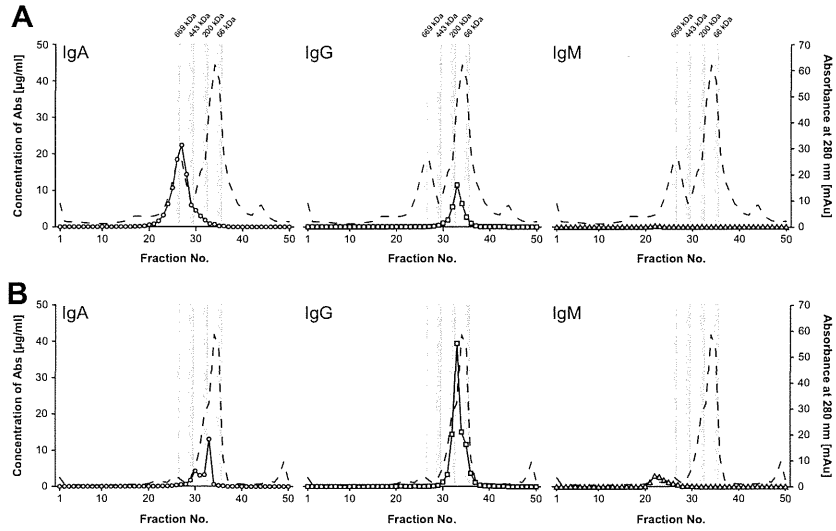

Fig. 2. Fractionation of nasal wash (**A**) and serum (**B**) samples from representative participants on Superose 6 columns. Quantification (µg/ml) of IgA (open circles), IgG (open squares), or IgM (open triangles) antibody levels and the absorbance at 280 nm (mAu, broken line) are shown. The grey zones in the upper part of the curves indicate the positions of the molecular weight markers [thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), and bovine serum albumin (66 kD)].

(Fig. 2B). Serum IgA (which showed a lower peak at about 360 kD in addition to a peak at about 150 kD) appeared to comprise both monomeric and dimeric IgA.

Taken together, the results of the fractionation analysis suggests that highly polymeric IgA is the predominant nasal antibody, and can be separated from nasal IgG and IgM. By contrast, the monomeric forms of IgG are the major component of total serum antibodies.

Neutralization Activity of the IgA and IgG Antibodies in The Nasal Wash and Serum Samples

To determine the isotype of the antibodies responsible for the neutralization activity induced by intranasal administration of the inactivated vaccine, nasal wash and serum samples from participant P1, who showed relatively high neutralization titres after the fifth vaccination, were separated on a Superose 6 column and the neutralization titre of the resulting antibody fractions assayed. The nasal polymeric IgA fraction (No. 27) showed a neutralization titre of 1:10, whereas the nasal monomeric IgG fraction (No. 33) showed a reciprocal neutralization titre of <1:10. However, the serum dimeric IgA fraction (No. 30) showed a neutralization titre of <1:10, whereas the serum peak monomeric IgG fraction (No. 33) showed a neutralization titre of 1:10 (Table IV). The respective peak fractions in the nasal wash were then concentrated to 100 µg/ml, and the neutralization activity of the nasal IgA antibodies (a mixture of fractions 26 and 27) was compared with that of the nasal IgG antibodies (a mixture of fractions 33 and 34). The nasal IgA fractions showed a neutralization titre of 1:40, whereas the nasal monomeric IgG fractions showed a neutralization titre of 1:10. Similarly, the neutralization activity of the serum IgA antibodies (100 µg/ml; a mixture of fractions 30 and 31) was compared with that of serum IgG antibodies (a mixture of fractions 33 and 34). The serum IgA fractions showed a neutralization titre of <1:10, whereas the serum

TABLE IV. Neutralization Titre of the IgA and IgG Fractions From the Nasal Wash and Serum Samples Following Separation on Superose Columns

	Neutralization titre[a]			
	Nasal wash		Serum	
	Polymeric IgA	IgG	Dimeric IgA	IgG
A/Uruguay (A/H3N2)				
Peak fraction: Separated on Superose column	10 (0)	<10 (<0)	<10 (<0)	10 (0)
Concentrated fraction (100 µg/ml)	40 (2)	10 (0)	<10 (<0)	10 (0)

The samples were collected from a representative subject vaccinated five times with an interval of 3 weeks between vaccinations.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

IgG fractions showed a neutralization titre of 1:10 (Table IV).

The peak polymeric IgA fraction (about 600 kD) from the nasal wash samples, as measured using an IgA ELISA, contained no IgG antibodies when measured using an IgG ELISA; however, the peak monomeric IgG fractions (about 150 kD) from the nasal wash comprised about 1/4 of IgA (data not shown). By contrast, about 1/10 of the peak dimeric IgA (about 380 kD) from the serum samples comprised IgG antibodies, whereas about 1/10 of the peak monomeric IgG fractions from the serum comprised IgA (data not shown). This suggests that nasal polymeric IgA is responsible for the neutralization activity observed in the peak polymeric IgA fractions (about 600 kD) from the nasal wash samples. Serum monomeric IgG appears to be responsible for the neutralization activity observed in the peak monomeric IgG fractions (about 150 kD) from the serum, because the IgA content of the IgG fractions was very small. In those nasal monomeric IgG fractions that contained a relatively high amount of IgA, both IgG and IgA may be responsible for the neutralization activity. Taken together, these results show that the main neutralizing antibody in the nasal mucus is highly polymeric IgA, while the main neutralizing antibody in the serum is monomeric IgG.

DISCUSSION

In the present study, neutralizing antibody responses and their properties were examined in nasal and serum samples from healthy adults after intranasal administration of a concentrated, inactivated split A/Uruguay (H3N2) vaccine (containing 45 μg HA per dose). The first intranasal administration of a concentrated split vaccine in young adults was conducted by Kuno-Sakai et al. [1994] and showed that both serum HI- and nasal HA-specific IgA antibodies were induced after two aerosol vaccinations, which protected against a challenge infection with a cold-adapted live virus vaccine. In the present trial, neutralizing antibody responses were examined in both serum and nasal wash samples obtained from adults given five doses of vaccine, with an interval of 3 weeks between doses. The nasal wash samples were concentrated to ensure that nasal and serum neutralization titres were assayed at equivalent levels (Table I).

To measure the concentration of IgA and IgG antibodies in the concentrated nasal wash samples, the standardized nasal wash samples were adjusted to 1 mg/ml of total protein, and contained about 1/10 amount of IgA and IgG found in natural nasal mucus [Kurono and Mogi, 1987]. Previous studies show that the total amounts of IgA and IgG increase between pre-vaccination and post-vaccination in BALB/c mice [Tamura et al., 1990, 2010]; however, the results of the present study show that the amount of total IgA (and other antibodies) recovered from the nasal mucus showed small variations at each sampling time, although this was not related to vaccination status (data not shown). Even allowing for small variations in the recovery of total IgA and IgG from the nasal mucus of each subject, the neutralization titres in the standardized nasal wash samples after vaccination appeared to be a reasonable reflection of the absolute antibody titre in the nasal mucus.

A $\geq$4-fold increase in the nasal neutralization titre was observed after the second vaccination in the four younger subjects, whereas a rise in the serum neutralization titre was observed only after the fifth vaccination in the three younger subjects (Table II and Fig. 1). Intranasal administration of a vaccine tends to induce inferior serum antibody responses, but superior nasal IgA responses, compared with intramuscular injection [Atmar et al., 2007]. The present study also showed that neutralization titres correlated well with HI titres, although the HI titres were lower than the corresponding neutralization titres (Table III). This result confirms the work of Okuno et al. [1990], who showed that HI titres are sometimes lower than the corresponding neutralization titres, depending on the strain of influenza A or B virus used in the HI assay.

Healthy adults who had already acquired immunity to influenza viruses due to previous natural infections or vaccinations (seropositive adults) showed both nasal and serum antibody responses induced by the nasal vaccine (Tables II and III, and Fig. 1). Clinical trials show that intranasal administration of inactivated vaccines induces both mucosal and systemic antibody responses in seropositive adults [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The induction of antibody responses in seropositive people by the nasal vaccine can be explained by the notion that the seropositive people have immunological memory for influenza viruses. Previous reports show that administration of an intranasal split vaccine plus adjuvant induces both local and systemic antibody responses in naive mice, and that the adjuvant is not required for a booster dose to induce an enhanced anamnestic immune response 4 weeks later [Tamura et al., 1989, 1992]. Administration of an adjuvant together with the vaccine stimulates innate immunity via several classes of pattern-recognition receptors (such as Toll-like receptors), which leads to the acquisition of specific immune responses, including immunological memory [Tamura et al., 1991, 2005; Tamura and Kurata, 2004].

Analysis of nasal wash and serum samples after passage through Superose 6 columns showed that the major component of nasal mucus antibodies was highly polymeric IgA, while that of serum antibodies was IgG (Fig. 2). In those subjects that received five doses of the intranasal A/Uruguay (H3N2) vaccine, the highly polymeric nasal IgA fractions were responsible for the majority of the neutralizing activity, whereas

the serum IgG fractions were responsible for the majority of the neutralizing activity in the serum (Table IV). These data are in agreement with those obtained in a previous mouse model experiment, in which IgA antibodies with neutralizing activity purified from the respiratory tract of mice immunized intranasally with HA molecules from the A/Puerto Rico/8/34 (H1N1) virus were polymeric, whereas the purified IgG antibodies with neutralizing activity were monomeric [Tamura et al., 1990]. Further study of the detailed structure of IgA, which has higher MW than expected for dimeric IgA [Song et al., 1995] remains to be performed.

Previous studies show that IgA in the respiratory tract is more cross-reactive with variant influenza viruses than IgG [Tamura et al., 1990, 1991]. This cross-reactivity seems to depend on the polymeric nature of IgA [Taylor and Dimmock, 1985; Palladino et al., 1995]. Taken together, these data suggest the potential for intranasally administered inactivated vaccines to induce cross-protection against antigenic variants of viruses in pre-immunized adults.

Both serum and mucosal HA-specific ELISA antibody responses after nasal vaccination need to be examined and compared with the corresponding neutralization and HI titres. In addition, neutralizing antibody responses to other influenza vaccines (from different strains, different subtypes or types of viruses, and from different forms of vaccines such as subvirion and whole virus vaccines) after nasal vaccination remain to be examined to compare the efficacy of nasal vaccines with that of the parenteral vaccine. Some of these studies are ongoing.

In conclusion, intranasal administration of an A/Uruguay split vaccine containing 45 μg HA resulted in induced nasal and serum neutralizing antibody responses in four out of five healthy adult subjects, with a neutralization titre of >1:40 after the second and the fifth administrations, respectively. These neutralizing antibody responses were largely due to the induction of nasal polymeric IgA and serum monomeric IgG.

ACKNOWLEDGMENTS

We express our appreciation to all the study participants and thank the authors whose work is cited in this paper. We would also like to thank Mr Patrick Costigan for proofreading.

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