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

Weeks (vaccination)	Neutralization titre against A/Uruguay virus (H3N2)[a]									
	P1		P2		P3		P4		P5	
	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.

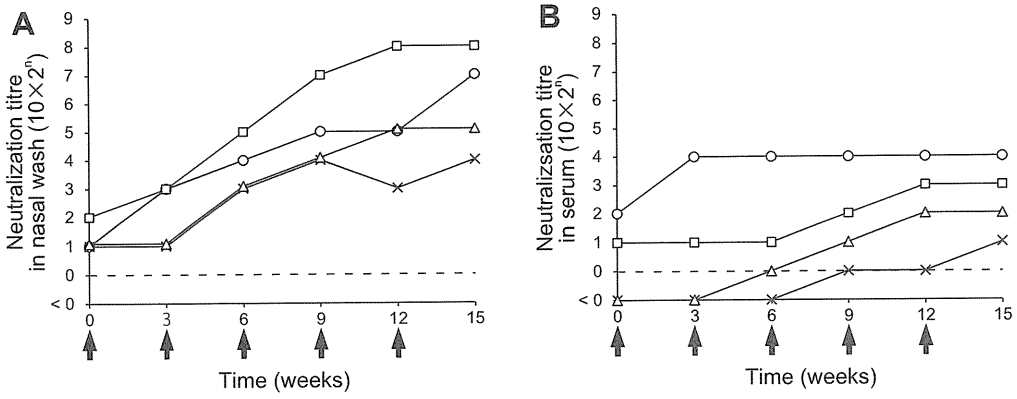

Fig. 1. Nasal wash and serum sample neutralization titres against A/Uruguay (H3N2) (pre- and post-intranasal immunization). Neutralization titres against the A/Uruguay virus in nasal washes (**A**) and serum (**B**) were determined in samples collected from four participants (18–60 years old; P1, open 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

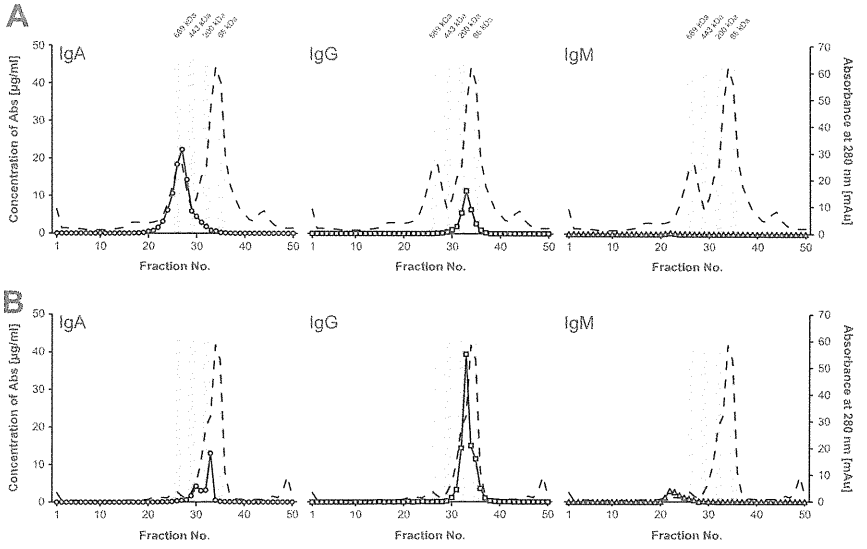

Fig. 2. Fractionation of nasal wash (**A**) and serum (**B**) samples from representative participants on Superose 6 columns. Quantification (μg/ml) of IgA (open circles), IgG (open squares), or IgM (open triangles) antibody levels and the absorbance at 280 nm (mAu, broken line) are shown. The grey zones 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 ≥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.

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Review

Mucosal IgA responses in influenza virus infections; thoughts for vaccine design

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ABSTRACT

The current challenge in influenza vaccine design is to induce long-lasting protection not only against the vaccine strain, but also against drifted (point mutations in the surface antigens HA or NA) and even shifted (exchange of genome segments) strains. Several immune mediators that can induce cross-protection have been described, such as CD4 T-cells, CD8 T-cells and antibodies, including IgA. However, most vaccines are now administered intramuscularly or subcutaneously and subsequently relatively little is known on the role of local, mucosal responses. Since local IgA responses have been shown to play an important role in responses to natural infection, and IgA responses in mice were shown to also be involved in cross-protection, the research on mucosal influenza vaccines is currently expanding. However, the functioning of the mucosal immune system, especially in the respiratory tract, is just beginning to be revealed. Here, the current knowledge on the induction of IgA, the role of influenza specific IgA producing B-cells in anti-influenza immunity as well as the role of humoral memory responses induced upon vaccination will be reviewed.

Contents

1. Introduction

Seasonal influenza A virus infections cause millions of cases each year with the highest risk of complications in very young and very old people as well as immunocompromised patients, all lacking a strong immune response. In addition, also more infectious or pathogenic strains can infect people, such as the 2009 pandemic influenza A virus (A(H1N1)pdm09), or highly pathogenic avian influenza A H5N1 virus, respectively. In contrast to seasonal influenza viruses, highly pathogenic strains can be more threatening for young, healthy people in whom tissue damage can be the result of overly powerful host inflammatory responses [1].

In the case of both seasonal and newly evolved strains, the most efficient way to fight the disease is preventing it by means of vaccination. However, current influenza vaccines are effective against a single type of influenza only, thus for the seasonal vaccine necessitating the presence of multiple strains, as well as a yearly renewal of the vaccine. In addition to the possibility of a mismatch of the vaccine with the actual circulating influenza strains, newly evolved strains, such as H5N1 and A(H1N1)pdm09, highlighted the need for improved cross-protection. Ideally, a universal influenza vaccine would be developed, that induces a strong and long lasting memory response which is cross-protective to drift variants as well as across subtypes of the influenza virus (Fig. 1). Many factors are

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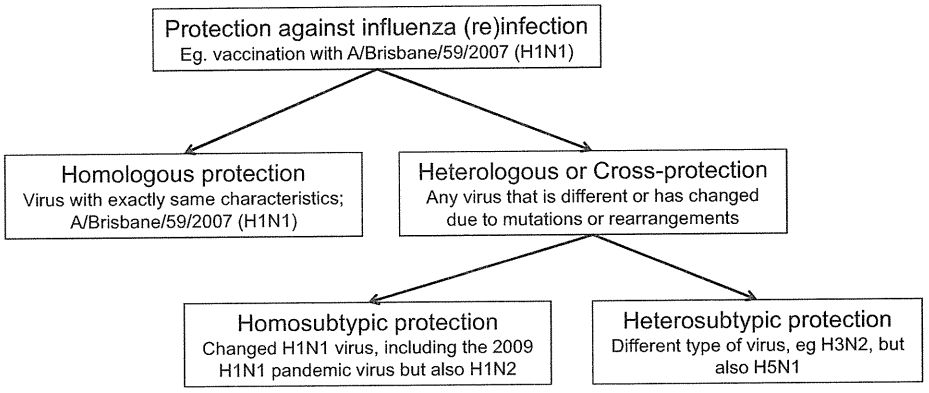

Fig. 1. Overview of different terms related to different types of protection. Since no official definition is available for these terms, this figure shows how these terms are used in this review. The definitions are based on the type of haemagglutinin antigen that the virus contains. For each term some 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

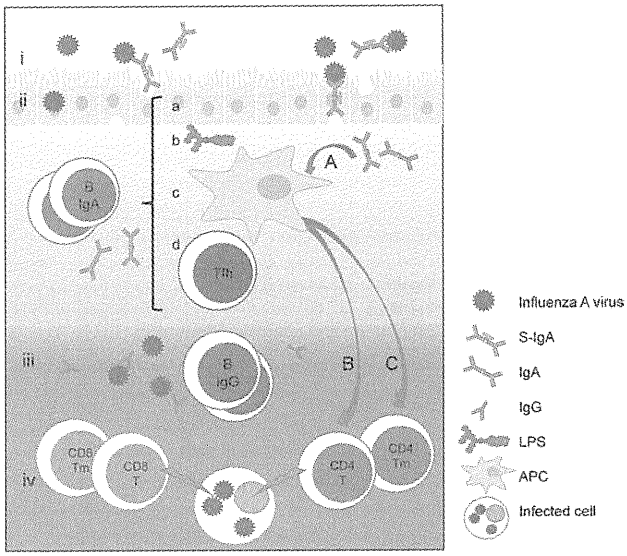

Fig. 2. The role of IgA in protection from influenza infection; function, induction and interaction. Function (i–iv): IgA can neutralize influenza viruses at the mucosal interface (i), even before they actually enter the host by crossing the mucosal barrier, thus existing IgA is the first line of defence 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.

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Identification of *Mycoplasma pneumoniae* type 2b variant strains in Japan

Mycoplasma pneumoniae is an important causative pathogen of community-acquired pneumonia, particularly among children and young adults. Two major groups of this bacterium, subtypes 1 and 2, are known and the major differences between these groups are variations in the *p1* gene, encoding P1 cytadhesin, an essential factor for the pathogenicity of this bacterium (Nakane *et al.*, 2011; Razin & Jacobs, 1992). The variations are observed in two parts of the *p1* gene, designated RepMP4 and RepMP2/3. These regions serve as targets for PCR-restriction fragment length polymorphism (RFLP) analysis (Cousin-Allery *et al.*, 2000) in genotyping studies (Fig. 1a).

In the present study, we genotyped the *p1* gene of 48 *M. pneumoniae* strains deposited at the Kanagawa Prefectural Institute of Public Health. These strains were isolated from pneumonia patients in Japan from 2004 to 2005. Thirty-nine of these strains were genotyped as subtype 1, and six strains were identified as variant 2a; however, the remaining three strains (designated T-103, Y-135 and Y-169) exhibited a slightly different PCR-RFLP pattern compared to the known strains. The genotyping pattern of strain Y-169 is shown in Fig. 1b next to the subtype 1, 2 and variant 2a strains M129, FH and 309, respectively. In the typing of the RepMP4 region (Fig. 1b, lanes 1–4), the pattern of strain Y-169 was similar to that of strains FH and 309, suggesting a relation with the subtype 2 group. However, the RepMP2/3 pattern was slightly different from the known pattern for this gene region (Fig. 1b, lanes 5–8). We sequenced the *p1* gene of strains T-103, Y-135 and Y-169 and confirmed that strains Y-135 and Y-169 harboured a *p1* gene with an identical sequence. The *p1* gene of strain T-103 was also identical to the other two strains except for one additional AGT trinucleotide at the variable-number tandem-repeat (VNTR) site (Zhao *et al.*, 2011; Fig. 1a). We compared the *p1* gene sequences published thus far with those derived in this study and found that a partial *p1* sequence reported by Dumke *et al.* (2006) (GenBank accession number DQ383277) was identical to the *p1* gene sequences determined here. The partial *p1* gene sequence of Dumke *et al.* (2006), classified as variant 2b, covered the 5′ half of the *p1* variation site determined in the

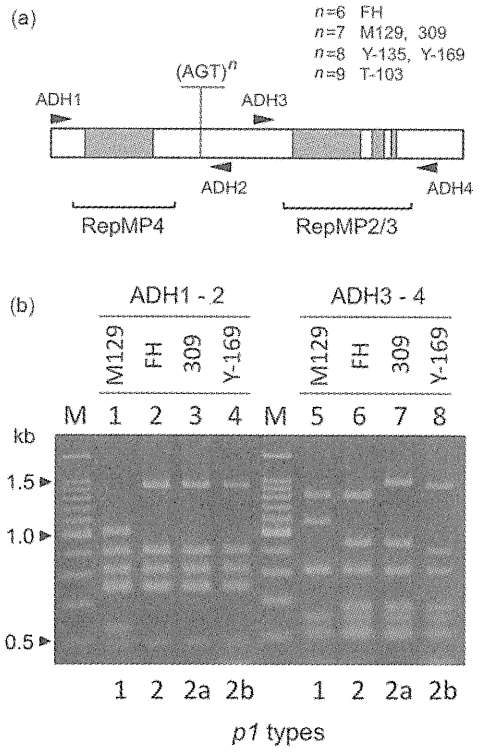

Fig. 1. PCR–RFLP typing of the *p1* gene. (a) Schematic illustration of the *p1* gene. Filled triangles indicate the binding sites of the PCR primers designed to amplify target sequences for RFLP typing (Cousin-Allery *et al.*, 2000; Kenri *et al.*, 1999). Shaded boxes in the *p1* gene indicate variation sites between subtypes. Areas of repetitive elements (RepMP4 and RepMP2/3 regions) are indicated by square brackets. Sequences similar to these RepMP regions are present in the *Mycoplasma pneumoniae* genome in multiple copies (Schwartz *et al.*, 2009; Wenzel & Herrmann, 1988). $(AGT)^n$ indicates a trinucleotide VNTR site (Zhao *et al.*, 2011). The number of these repeats (n) for selected strains is shown in the upper right corner. (b) PCR–RFLP typing patterns of selected strains. Typing patterns were obtained by *Hae*III digestion of PCR products obtained using ADH1 and 2 (lanes 1–4) and ADH3 and 4 (lanes 5–6) primer sets. *Hae*III-digested fragments were visualized by 2% agarose gel electrophoresis and ethidium bromide staining. Strain names analysed are labelled above the lane numbers.

The DDBJ/EMBL/GenBank accession numbers for the variant 2b *p1* gene sequences of strains T-103, Y-135 and Y-169 are AB691539, AB691540 and AB678699, respectively.

present study. Recently, another partial *p1* gene sequence similar to that reported by Dumke *et al.* (2006) was reported in the USA (Schwartz *et al.*, 2009; Spuesens *et al.*, 2010a). This sequence (GenBank GQ861494), obtained from a strain named isolate 3, matched the entire variation site of the *p1* gene sequence determined here, except for a single base-pair mismatch (C→T at nucleotide position 4164 of the *p1* gene sequence of strain Y-169). From these results, we concluded that the *p1* gene sequences of strains T-103, Y-135 and Y-169 corresponded to that of variant 2b. By using standard PCR-RFLP genotyping we were able to detect *p1* gene sequences corresponding to variant 2b (Fig. 1b, lane 8). Except for the variant 2b-specific sequence in the RepMP2/3 region and the number of AGT trinucleotides at the VNTR site, the full-length *p1* gene sequences of variant 2b were highly similar to subtype 2 and variant 2a (strains FH and 309, respectively; GenBank CP002077 and AP012303; Kenri *et al.*, 2012; Krishnakumar *et al.*, 2010) with only two single-nucleotide polymorphisms compared with strain FH and seven single-nucleotide polymorphisms compared with strain 309.

Sequence variations in the *p1* gene are thought to be generated by DNA recombination events between the *p1* locus and repetitive sequences similar to the RepMP4 and RepMP2/3 regions (RepMP elements) in the *M. pneumoniae* genome (Kenri *et al.*, 1999; Spuesens *et al.*, 2009; Wenzel & Herrmann, 1988). The region of the *p1* gene containing sequence variations in variant 2b is about 640 bp in length. This 640 bp sequence is identical to a RepMP element outside the *p1* locus of the strain FH genome (position 128399–129039), suggesting variant 2b variation was derived from this sequence. To date, four variants of the *p1* gene in subtype 2 are known, including variants 2a, 2b and 2c, as well as the *p1* gene of strain Mp3896. Variant 2c was recently reported in the Netherlands and in China (Spuesens *et al.*, 2010b; Zhao *et al.*, 2011). Variant 2c exhibits variations at the RepMP4 region, although the RepMP2/3 region of this variant is almost identical to that of variant 2a. Two slightly different variant 2c sequences were reported in China (GenBank JN048894 and JN048895; Zhao *et al.*, 2011). Strain Mp3896 was isolated in France (Pereyre *et al.*, 2007) and could not be classified by PCR-RFLP. The *p1* gene of this strain has unique variations in both the RepMP4 and RepMP2/3 regions (GenBank EF656612); however, it is now believed that this sequence was created by DNA recombination in the genetic background of the subtype 2 group (Spuesens *et al.*, 2009). Only a single strain of this variant has been reported so far. In contrast to subtype 2, subtype 1 variants are rarely detected; only one variant (strain Mp4817, GenBank AF290000) has been reported to date (Dorigo-Zetsma *et al.*, 2001). The scarcity of subtype 1 variants may be linked to a reduced recombination activity in subtype 1 strains compared with subtype 2 (Krishnakumar *et al.*, 2010).

In the present study, we identified three *M. pneumoniae* strains harbouring variant 2b *p1* genes for what is believed to be the first time in Japan. These strains were collected from two distant areas (Y-135 and Y-169 were isolated at Yamagata prefecture in 2004 and T-103 was isolated from Kanagawa prefecture in 2005) and variant 2b strains are rarely detected in Japan at present. This situation is similar to other areas of the world. Variant 2b sequences were originally identified in central Europe by molecular methods (Dumke *et al.*, 2006). In Germany, a relatively higher detection rate of variant 2b DNA was reported (5–15 % in specimens collected between 2003 and 2006); however, the bacterial strain has not been isolated (Dumke *et al.*, 2010). A single variant 2b isolate was reported in the United States (Schwartz *et al.*, 2009). Presently, variant 2b strains might constitute a minor proportion of clinically relevant strains; however, it is possible that they will become a major variant of clinical strains in the future, considering that the variant 2a strains that were rarely detected in 1990s are now commonly found worldwide (Dumke *et al.*, 2010; Kenri *et al.*, 2008; Zhao *et al.*, 2011). Therefore, monitoring of variant 2b strains is needed to attain a better understanding of the epidemiology and evolutionary aspects of *M. pneumoniae* infection.

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CONCISE REPORT

Impact of tocilizumab therapy on antibody response to influenza vaccine in patients with rheumatoid arthritis

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ABSTRACT

Objectives We assessed the influence of tocilizumab (TCZ), a humanised monoclonal anti-interleukin-6 receptor antibody, on antibody response following influenza vaccination in patients with rheumatoid arthritis (RA).

Methods A total of 194 RA patients received inactive trivalent influenza vaccination (A/H1N1, A/H3N2 and B/B1 strains). All patients were classified into the TCZ (n=62), TCZ+methotrexate (MTX) (n=49), MTX (n=65) and RA control (n=18) groups. Antibody titres were measured before and 4–6 weeks after vaccination using the haemagglutination inhibitory assay.

Results For the A/H1N1 and A/H3N2 strains, the TCZ and TCZ+MTX groups achieved fold increases of 9.9–14.5, postvaccination seroprotection rates greater than 70% and seroresponse rates greater than 40%. For the B/B1 strain, seroresponse rates were approximately 30%, but fold increases and seroprotection rates were 5.0–5.4 and greater than 70%, respectively, in these treatment groups. MTX had a negative impact on vaccination efficacy, but adequate responses for protection were nevertheless demonstrated in the MTX group. Neither severe adverse effects nor RA flares were observed.

Conclusions TCZ does not hamper antibody response to influenza vaccine in RA patients. Influenza vaccination is considered effective in protecting RA patients receiving TCZ therapy with or without MTX.

INTRODUCTION

Influenza vaccination is the most effective method for preventing influenza virus infection and its potentially severe complications. Patients with rheumatoid arthritis (RA) are at an increased risk for infectious diseases due to the nature of RA and its treatment with immunosuppressive agents;[1] therefore, this patient population is a potential candidate for influenza vaccination. Treatment with antitumour necrosis factor α (anti-TNFα) agents may impair antibody response to influenza vaccination in patients with RA and other rheumatic diseases, but the response is large enough to warrant influenza vaccination for such patients.[2–8]

Tocilizumab (TCZ), a humanised monoclonal interleukin-6 (IL-6) receptor antibody, is effective in the treatment of patients with moderate to severe RA who have shown inadequate responses to methotrexate (MTX) and one or more anti-TNFα agents.[9] Our concern is the impact of TCZ on protective antibody response to influenza vaccination because IL-6 was originally identified as a factor that plays an essential role in terminal differentiation of B cells into antibody producing plasma cells.[10] Data regarding the efficacy and safety of influenza vaccination are lacking in RA patients receiving TCZ. Only one attempt at evaluating the efficacy of influenza vaccine has so far been made in a small number of paediatric patients receiving TCZ therapy for systemic onset juvenile idiopathic arthritis.[11]

To address this issue, we determined antibody response to trivalent inactivated influenza vaccine in RA patients being treated with TCZ, MTX or both agents, and compared parameters for efficacy of vaccination among these groups.

METHODS

Patients

RA patients aged 18 or older who had been receiving TCZ (an intravenous infusion of 8 mg/kg every 4 weeks) for at least 4 weeks and/or MTX (6–18 mg per week) for 12 weeks or more at our rheumatology outpatient clinics were invited to participate in this open-label study. RA patients who had been receiving bucillamine or salazosulphapyridine were also included as RA controls. All participants fulfilled the 1987 American College of Rheumatology criteria for diagnosis of RA. Exclusion criteria were current use of 10 mg/day or more of prednisolone, current use of tacrolimus or leflunomide, a recent history (within 3 months) of influenza infection, and a recent history (within 6 months) of influenza vaccination.

Vaccine

We used commercially available inactivated trivalent influenza vaccine (Biken HA, Mitsubishi Tanabe Pharm Corporation, Osaka, Japan) containing 30 μg of purified haemagglutinin of each of the following: A/California/7/2009 (H1N1)-like strain (A/H1N1 strain), A/Victoria/210/2009 (H3N2)-like strain (A/H3N2 strain) and B/Brisbane/60/2008-like strain (B/B1 strain). Patients received a single dose of vaccine (0.5 ml) subcutaneously from October 2011 until January 2012. For RA patients receiving TCZ, the vaccination was done on the same day as TCZ infusion.

HI tests

Sera were collected immediately before and 4–6 weeks after vaccination. For the detection of

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influenza antibodies, haemagglutination inhibition (HI) tests were performed in duplicate at SRL (Tachikawa, Tokyo, Japan), according to WHO standard procedure using haemagglutinin antigens representing all three strains that were included in the vaccine. Geometric mean titres (GMTs) of HI antibodies before and after vaccination, and fold increases relative to prevaccination titres (geometric means of postvaccination to prevaccination antibody titre ratios) were determined. GMTs were calculated from log-transformed values of HI antibody titres. For statistical analysis, a titre of 5 was arbitrarily assigned to sera with undetectable titres of <10. Seroprotection was defined as antibody titres of ≥40. Seroconversion was defined as postvaccination antibody titres of ≥40 in patients whose prevaccination titres were <10. Seroresponse was defined as seroconversion or fold increases in antibody titres of ≥4 in patients whose prevaccination titres were ≥10.

Monitoring adverse effects and disease activity

Systemic adverse events and worsening of RA occurring 4–6 weeks after vaccination were recorded. Systemic adverse effects included fever, tiredness, sweating, myalgia, chills, headache, arthralgia, diarrhoea and common cold-like symptoms. RA activity was monitored using a disease activity score for 28 joints and a clinical disease activity index.

Statistical analysis

In univariate analyses for categorical variables, differences between treatment groups were analysed using the χ^2 test or Fisher's exact probability test. Continuous variables were assessed by the Mann–Whitney U test for comparisons of non-parametric data between the two treatment groups, and analysis of variance with post hoc Tukey's honestly significant difference test for comparisons of parametric data between the four treatment groups. A paired-sample t test was used to compare differences in GMTs between prevaccination and postvaccination.

For all tests, probability values (p values) <0.05 were considered to indicate statistical significance. All calculations were performed using Excel Statistical Analysis 2008 (SSRI Co., Tokyo, Japan) or PASW Statistics V.18 (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Clinical and demographic characteristics of participants

A total of 194 RA patients were classified into four groups according to their ongoing anti-RA therapy. One group of 62 patients was treated with TCZ as a monotherapy (TCZ group); 65 patients were treated with MTX alone (MTX group); 49 patients received a combination therapy consisting of TCZ and MTX (TCZ+MTX group); and 18 patients received bucillamine or salazosulphapyridine monotherapy (RA control group). Clinical and demographic characteristics are shown in table 1.

Antibody titres

After vaccination, GMTs for all strains were increased significantly. Regarding the A/H3N2 strain, a significantly higher post-GMT was obtained in the TCZ group compared with that in the MTX group (p=0.009) (table 2). The TCZ group also showed a higher post-GMT for the B/B1 strain than did the MTX group and the RA control group (p=0.044 and p=0.031,

Table 1 Clinical and demographic characteristics of RA patients prior to influenza vaccination

	MTX group (n=65)	TCZ+MTX group (n=49)	TCZ group (n=62)	RA control (n=18)	p Values between treatment groups
Male/female	11/54	5/44	11/51	3/15	NS
Age, years, mean (95% CI)	67 (65.0 to 68.9)	62.9 (59.8 to 65.9)	65.2 (61.6 to 68.8)	67.3 (62.3 to 72.4)	NS
Prior influenza vaccination, number of patients (%)	47 (72.3)	36 (73.5)	50 (80.6)	12 (66.7)	NS
RA duration, years, mean (95% CI)	9.8 (7.7 to 11.9)	7.5 (5.8 to 9.2)	14.6 (11.5 to 17.7)	11.1 (4.8 to 17.4)	0.029 (M vs T) 0.001 (T/M vs T)
MTX dose, mg/week, median (25th, 75th percentiles)	8 (6, 8)	8 (6, 8)	–	–	NS
MTX duration, months, median (25th, 75th percentiles)	58 (17, 78)	54 (29, 89)	–	–	NS
TCZ duration, weeks, median (25th, 75th percentiles)	–	68 (24, 104)	64 (21, 107)	–	NS
Use of prednisolone, number of patients (%)	13 (20)	12 (24.5)	22 (35.5)	1 (5.6)	0.016 (T vs C)
Prednisolone dose, mg/day, mean (95% CI)	0.87 (0.4 to 1.34)	0.90 (0.33 to 1.47)	1.02 (0.54 to 1.49)	–	NS
Positive RF, number of patients (%)	38 (58.5)	42 (85.7)	46 (74.2)	7 (38.9)	0.002 (M vs T/M) 0.0001 (T/M vs C) 0.005 (T vs C)
Positive anti-CCP Abs, number of patients (%)	46 (70.8)	43 (87.8)	56 (90.3)	6 (33.3)	0.030 (M vs T/M) 0.006 (M vs T) 0.004 (M vs C) <0.0001 (T/M vs C) <0.0001 (T vs C)
CDAI (25th, 75th percentiles)	5.3 (3.7–7.8)	6.2 (4.5–7.8)	9.5 (7.9–11.1)	8.2 (4.8–11.5)	0.001 (M vs T) 0.027 (T/M vs T)
Lymphocytes, /μl, mean (95% CI)	1368 (1237 to 1500)	1395 (1255 to 1535)	1622 (1500 to 1744)	1478 (1098 to 1857)	0.038 (M vs T)

Data were obtained immediately before influenza vaccination. Prior influenza vaccination represents that administered last season (2010/2011). p Values between treatment groups were determined by the Mann–Whitney U test, post hoc ANOVA using Tukey's HSD test, the χ^2 test or Fisher's exact probability test.
ANOVA, analysis of variance; anti-CCP Abs, anti-cyclic citrullinated peptide antibodies; C, RA control group; CDAI, clinical disease activity index; HSD, honestly significant difference; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; RF, rheumatoid factor; T, TCZ group; T/M, TCZ+MTX group; TCZ, tocilizumab.

Table 2 GMTs and fold increases of HI antibodies for three influenza strains in the RA treatment groups prior to and after influenza vaccination

	MTX group (n=65)	TCZ+MTX group (n=49)	TCZ group (n=62)	RA control group (n=18)	p Values between treatment groups
GMTs					
A/H1N1					
Before	31.7 (16.1–47.2)	59.5 (19.9–99.1)	62.0 (25.4–125.4)	15.3 (8.3–22.3)	NS
After	120.5 (75.3–165.6)*	162.1 (86–238.2)**	211.7 (142–281.4)*	169.4 (11.5–327.4)*	NS
A/H3N2					
Before	37.9 (15.5–60.4)	42.6 (25.2–59.9)	55.2 (31.8–78.7)	36.9 (11.9–62.0)	NS
After	120.2 (80.2–160.2)*	140.7 (82–199.4)***	237.8 (169.1–306.5)*	93.9 (54.1–133.6)**	0.009 (M vs T)
B/B1					
Before	45.5 (30.2–60.7)	43.2 (29.8–56.5)	72.1 (53.3–90.9)	23.9 (12.2–35.6)	0.017 (T vs C)
After	103.1 (74.9–131.3)*	105.1 (69.4–140.8)*	161.8 (123.8–144)*	68.9 (45.7–92.1)*	0.044 (M vs T) 0.031 (T vs C)
Fold increase					
A/H1N1	12.6 (5.8–19.5)	14.5 (7.2–21.9)	12.0 (9.8–17.7)	11.2 (3.0–19.4)	NS
A/H3N2	9.6 (5–14.2)	9.9 (5.2–14.6)	12.0 (6.6–17.3)	5.3 (2.7–8.0)	NS
B/B1	3.5 (2.5–4.4)	5.4 (2.4–8.3)	5.0 (3.3–5.7)	5.8 (3.1–8.4)	NS

Data are expressed as the mean (95% CIs). Differences between prevaccination and postvaccination GMTs were assessed using the paired-sample t test. Comparisons between the four treatment groups were performed by post hoc ANOVA using Tukey's HSD test.
*p<0.0001, **p=0.009 and ***p=0.001 based on comparisons with prevaccination titres.
ANOVA, analysis of variance; C, RA control group; GMT, geometric mean titre; HI, haemagglutination inhibition; HSD, honestly significant difference; M, MTX group; MTX, methotrexate; NS, not significant; RA, rheumatoid arthritis; T, TCZ group; TCZ, tocilizumab.

respectively). Fold increases in GMTs for the three strains were ≥3.5-fold in all treatment groups. These groups achieved similar levels of fold increases for each strain and there were no statistically significant differences.

Seroprotection, seroresponse and seroconversion rates

After vaccination, seroprotection rates for the three influenza strains were increased significantly in all treatment groups (figure 1A). The TCZ and TCZ+MTX groups achieved postvaccination protection rates of >70% for all the influenza strains. Regarding the A/H3N2 and B/B1 strains, postvaccination seroprotection rates were significantly higher in the TCZ group compared with those in the other three treatment groups (for A/H3N2, p<0.0005 vs MTX, p=0.001 vs TCZ + MTX p=0.006 vs RA control; for B/B1, p=0.007 vs MTX, p=0.023 vs TCZ +MTX, p=0.007 vs RA control). Seroprotection rates for the A/H1N1 strain were similar among all the groups tested.

For the A/H1N1 and A/H3N2 strains, seroresponse rates were >40% in the MTX, TCZ and TCZ+MTX groups, while the rates for the B/B1 strain in these groups were approximately 30% (figure 1B). The seroresponse rate for the A/H3N2 strain was significantly higher in the TCZ group compared with that in the MTX group (p=0.04). Seroconversion rates for the three influenza strains were greater than 40% in all treatment groups (figure 1C). The TCZ group showed a significantly higher seroconversion rate for the A/H3N2 strain than did the MTX group (p=0.032).

Predictive factors for seroresponse to influenza vaccination

In multivariate logistic regression analysis, TCZ use was not identified as the predictive factor for seroresponse to influenza vaccination (see online supplementary table S1). For the A/H3N2 strain, the negative association of current MTX use with seroresponse was confirmed (p=0.04). Prior influenza vaccination was negatively associated with seroresponse for all the three strains (for A/H1N1, p=0.006; for A/H3N2, p=0.01; for B/B1, p<0.0001). This may have reflected ceiling effects; that is, higher prevaccination protection rates may, at least in part, have influenced the observed seroresponse rates.

Vaccination safety

Neither systemic adverse effects nor exacerbation of RA was experienced by any patients during a follow-up period of 4–6 weeks after vaccination.

DISCUSSION

Antibody response to the A/H1N1 and A/H3N2 strains in the TCZ and TCZ+MTX groups met all three requirements of the European Medicines Agency (EMA) guidance for assessment of influenza vaccines specified by the Committee for Proprietary Medical Products (CPMP).[12] For the B/B1 strain, these treatment groups met two of the EMA/CPMP criteria. The MTX group fulfilled two of the EMA/CPMP criteria for all strains. Multivariate logistic analysis confirmed that TCZ use is not a predictive factor for inadequate antibody response for any influenza strain.

IL-6 works as a B cell differentiation factor, which induces activated B cells to produce immunoglobulin.[10] The blockage of IL-6 activity following TCZ therapy, therefore, would be expected to reduce humoral immune response to influenza vaccination. Kopf *et al*[13] indicated that T cell-dependent antibody response against virus infection is impaired in IL-6-deficient mice. Unlike anti-infliximab or antiadalimumab antibodies, anti-TCZ antibodies rarely developed in RA patients receiving 8 mg/kg of TCZ, even as monotherapy.[14 15] Nevertheless, the present study has clearly indicated that RA patients receiving TCZ therapy can be effectively and safely immunised with influenza vaccine. One possible explanation may be that, unlike rituximab, TCZ is not a B cell-targeting antibody that can induce B cell depletion. Given that a variety of cytokines are released from activated helper T cells, antibody production may not depend simply on IL-6. Costelloe *et al*[16] showed that IL-6 is not required for antigen (influenza virus)-specific antibody responses by non-fractionated tonsillar mononuclear cells or by T cell-depleted B cells in the presence of IL-2. Another explanation may be that IL-6 signalling is not inhibited completely in lymphoid tissue, locations in which vaccination-mediated immune response is initiated, even when maximum saturation of soluble IL-6 receptors in the circulation is achieved with