ORIGINAL PAPER

Intranasal delivery of whole influenza vaccine prevents subsequent allergen–induced sensitization and airway hyper–reactivity in mice

A. Minne*[†], J. Jaworska[†], K. Gerhold[†], B. Ahrens[†], A. Avagyan[†], R. Vanbever*, P. M. Matricardi[†], A. C. Schmidt[‡] and E. Hamelmann[†]

[†]*Department for Pediatric Pneumology and Immunology, Charité University Medicine, Berlin, Germany,* *Department of Pharmaceutical Technology, Université catholique de Louvain, Brussels, Belgium and [‡]Department of Neonatology, Charité University Medicine, Berlin, Germany*

Clinical and Experimental Allergy

Summary

Background Infection with influenza virus has been associated with seemingly opposing effects on the development of asthma. However, there are no data about the effects of mucosal vaccination with inactivated influenza on the inception of allergic asthma.

Objective To assess the immunological effects of inhaled inactivated influenza vaccine, using two different types of flu vaccines, on the inception of allergic sensitization and allergen-mediated airway disease in a mouse model.

Methods BALB/c mice were intranasally or intratracheally vaccinated with whole or split influenza virus vaccine (days −1 or −1, 27) before systemic sensitization with ovalbumin (OVA) (days 1, 14) and repeated airway allergen challenges (days 28–30). Allergen sensitization (IgE serum levels), airway inflammation (differential cells in bronchoalveolar lavage fluid) and airway hyper-reactivity (AHR) (*in vivo* lung function) were analysed.

Results The intranasal instillation of whole influenza vaccine before allergen sensitization significantly reduced the serum levels of total and OVA-specific IgE as well as allergen-induced AHR. Prevention was due to an allergen-specific shift from a predominant T helper (Th)2- towards a Th1-immune response. Application of split influenza vaccine did not show the same preventive effect.

Conclusion Intranasal administration of inactivated whole influenza vaccine reduced subsequent allergen sensitization and prevented allergen-induced AHR. Our results show that the composition of the influenza vaccine has a major influence on subsequent development of allergen-induced sensitization and AHR, and suggest that mucosal inactivated whole influenza vaccination may represent a step towards the development of a preventive strategy for atopic asthma.

Correspondence:
Eckard Hamelmann, Department for
Pediatric Pneumology and Immunology,
Charité University Medicine,
Augustenburger Platz 1, Berlin
D-13353, Germany. E-mail:
eckard.hamelmann@charite.de

Introduction

Over the last few decades, the number of patients affected by allergic diseases in the western world has considerably increased. Allergic asthma presents a high burden measured in terms of school and work absence, unplanned emergency care, hospitalization and even fatal outcomes. Major efforts to determine how to prevent the inception of allergic asthma have mostly been mostly unsuccessful so far. With reference to the well-known 'hygiene hypothesis', infections with distinct bacteria [1], helminths [2] or viruses [3] were found to be associated with a decreased risk of developing asthma, probably by maintaining the natural balance of the adoptive immune system through regulatory mechanisms [1, 4]. Consequently, it has been proposed that certain microbes or microbial compounds may be used in a safe way to prevent or treat allergy and asthma [5, 6].

As a quite well-established example of this theory, several epidemiological studies have shown that exposure to respiratory viruses such as the influenza virus, particularly early in life, seems to be protective against persistent wheeze or asthma [3, 7, 8], although infections with these viruses may cause acute wheezing or exacerbations of already-established bronchial asthma in infants and older children [9]. Interestingly, studies in animal models with

the replicating wild-type influenza virus have supported this hypothesis and suggest that the underlying mechanism for the protective effect lies in the induction of a local T helper (Th)1 response counterbalancing allergen-induced Th2 immune responses [10, 11].

We reasoned that inactivated but still immunogenic forms of the wild-type virus, which are currently licensed only as injectable vaccines, might maintain the capacity to protect from allergic sensitization and atopic asthma when administered directly to the airways. If this were true, then mucosal influenza vaccination could be safely tested in humans as a means to reproduce the protective effect of early childhood infections without the risk of airway inflammation (AI) associated with active viral replication in the respiratory tract. As there are, to our knowledge, no data available that may help to answer this important question, we aimed to examine the potential immunomodulatory effects of two types of inactivated influenza vaccines in a well-established mouse model for allergen-mediated sensitization and airway disease. We tested whole and split virion influenza vaccines, which differ in that the latter is prepared by fragmentation of the former, thus containing less viral RNA and lacking the viral lipid envelope [12]. We report here that intranasal instillation of a whole, but not of a split, influenza vaccine before allergen sensitization significantly reduced allergen-induced sensitization as well as AI and hyper-reactivity, and thus may serve as a novel approach for primary prevention of asthma.

Material and methods

Experimental protocol

Female BALB/c mice, 6–8 weeks of age, were purchased from Harlan Winkelmann (Borchen, Germany) and kept under pathogen-free conditions. All experimental procedures were approved by the institutional animal ethics committee.

Sensitization. On days 1 and 14, mice were systemically sensitized by intraperitoneal (i.p.) injections with 20 μg of ovalbumin (OVA) (Grade VI, Sigma, Munich, Germany) emulsified in 2 mg of aluminium hydroxide (AlumInject; Pierce, Rockford, IL, USA) as an adjuvant in a total volume of 200 μL (Table 1). Controls were placebo-sensitized with phosphate-buffered saline (PBS) instead of OVA. Alum itself did not induce Th2-immune responses (data not shown).

Challenge. All OVA-sensitized mice were challenged with aerosolized OVA (100 mg/10 mL, Grade V) on days 28, 29 and 30.

Flu priming. To investigate the effects of vaccination before allergen sensitization, slightly anaesthetized mice were intranasally (i.n.) exposed on day 1 to (A) a combination of three split antigen influenza virus strains [5 μg hemagglutinin (HA)/strain, A/Wyoming/3/2003, A/New Caledonia/20/99, B/Yiangsu/10/2003, kindly provided by GlaxoSmithKline Biologicals, Rixensart, Belgium] (split flu/OVA, *n* = 6), or (B) a combination of three whole inactivated influenza virus strains (5 μg HA/strain, A/Wyoming/3/2003, A/New Caledonia/20/99, B/Yiangsu/10/2003, GlaxoSmithKline Biologicals) (whole flu/OVA, *n* = 12) (Table 1).

Flu boost. To investigate the effects of vaccination before allergen sensitization and challenge, mice were primed with vaccines as described above and additionally received on day 27 an intratracheal booster immunization with a combination of split antigen influenza virus from the same strains (1.5 μg HA/strain) (whole flu/OVA/split flu, *n* = 12) (Table 1). Elsewhere, we have performed analyses of repeated vaccination with whole and split influenza virus and found no induction of AI by the vaccine alone (article in process). Negative controls were placebo-vaccinated and placebo-sensitized/challenged with PBS (PBS, *n* = 8). Controls for effects of vaccination were placebo-primed and boosted with PBS, before OVA

Table 1. Sensitization and immunization protocol

Group	Flu priming (day −1)	OVA sensitization (days 1, 14)	Flu boost (day 27)	OVA challenge (days 28–30)
Negative control: PBS	PBS	PBS	PBS	PBS
Positive control: PBS/OVA	PBS	OVA	PBS	OVA
Split flu/OVA	Split flu	OVA	PBS	OVA
Whole flu/OVA	Whole flu	OVA	PBS	OVA
Whole flu/OVA/split flu	Whole flu	OVA	Split flu	OVA

On day −1, BALB/c mice were intranasally immunized with whole or split influenza vaccine or sham immunized with saline (PBS). On days 1 and 14, mice were systemically sensitized by intraperitoneal injections with 20 μg of OVA in alum or sham sensitized with PBS. One group of mice (whole flu/OVA/split flu) received an intratracheal booster immunization with split influenza vaccine on day 27. All OVA-sensitized groups were challenged with aerosolized OVA on days 28, 29 and 30.
OVA, ovalbumin; PBS, phosphate-buffered saline.

sensitization and challenge, respectively (PBS/OVA, $n = 14$). Controls for the effect on OVA sensitization by trace amounts of OVA present in the vaccine preparations demonstrated no suppressive or tolerogenic effects ($n = 4$, data not shown).

Serum levels of total and ovalbumin-specific immunoglobulins

On day 32, the serum levels of total and OVA-specific Ig (IgE, IgG1, IgG2a) were measured by ELISA, as described previously [13].

Bronchoalveolar lavage

On day 32, lungs were lavaged twice with 0.8 mL PBS. Cells of both aliquots were pooled; cytospin slides were stained with Diff Quik (Dade Behring AG, Marburg, Germany). Cells were differentiated by morphological criteria by counting 200 cells under light microscopy.

Histology

Following bronchoalveolar lavage (BAL), the lungs were instilled with 1 mL 50% TissueTek (Reichart-Jung, Nu-βloch, Germany) in PBS. The left lobe of the lungs was removed, embedded in TissueTek cryomatrix and frozen by rapid immersion in liquid nitrogen. Frozen lungs were maintained at $-70\,°C$. Sections (8 μm thick) were obtained by means of a microtome. Sections were stained with haematoxylin–eosin, coverslipped and examined by light microscopy.

In vivo *airway reactivity*

On day 31, *in vivo* lung function was determined by whole-body plethysmography (EMKA Technologies, Paris, France), as reported previously [14]. Animals were exposed to aerosolized PBS for baseline reading and then to increasing concentrations of methacholine (MCh) (6–50 mg/mL). Airway reactivity (AR) was expressed as an increase of enhanced pause values for each concentration of MCh relative to baseline Penh values.

Specific proliferation

On day 32, the proliferative responses of spleen mononuclear cells (MNCs) cultured with medium, Concanavalin A (ConA; 2.5 μg/mL) or OVA (Grade VI, 50 μg/mL) were determined by ^{3}H-thymidine incorporation (0.5 μCi/200 μL, Amersham Buchler, Braunschweig, Germany) as reported previously [15].

In vivo *cytokine production*

On day 32, MNCs and peribronchial lymph node (PBLN) cells were isolated and cultured with phorbol-12-myristate-13-acetate and Ionomycin (PMA; 10 ng/mL, Ionomycin; 1 μg/mL) or OVA (50 μg/mL). Levels of cytokines were assessed in cell culture supernatants of spleen and PBLN MNC by ELISA, as described previously [13, 15]. Levels of IFN-γ and IL-10 were assessed by Pharmingen OptEia kits (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Groups of mice were comprised of six to 14 animals. Data were analysed using the JMP version 4.0.2 and GraphPad Prism version 4.00 software programs. Values for all measurements were expressed as mean ± standard error of the mean. Pairs of groups were compared by one-way ANOVA with Tukey's multiple comparison post test. Statistical significance was set at $P < 0.05$.

Results

Effect of influenza vaccination on allergen-induced sensitization and airway disease

Immunoglobulin production. OVA sensitization induced a highly significant increase in the serum levels of total and OVA-specific IgE compared with negative controls. This humoral response to allergen was significantly inhibited in mice treated with whole flu vaccine before first OVA-sensitization (whole flu/OVA), as opposed to other flu treatments (Fig. 1a). The split flu/OVA group showed only a non-significant trend towards reduced total and OVA-specific IgE levels, while the additional booster vaccination of already sensitized mice before airway allergen challenges (whole flu/OVA/split flu) reversed the decrease in IgE levels observed after priming with vaccine, and significantly enhanced OVA-specific IgE levels when compared with the latter (Fig. 1a). Further, OVA sensitization induced a sharp increase in the production of OVA-specific IgG1 and IgG2a serum levels compared with non-sensitized controls. Interestingly, OVA-specific IgG2a serum levels were significantly increased in whole flu, but not split flu, vaccinated OVA-sensitized mice, as compared with positive control (PBS/OVA) (Fig. 1b). IgG1 levels were gradually decreased after vaccination with split and whole flu, and this trend was even more marked in the mice vaccinated before and during sensitization (whole flu/OVA/split flu). This latter group also showed no increase in IgG2a levels compared with whole flu/OVA mice (Fig. 1b).

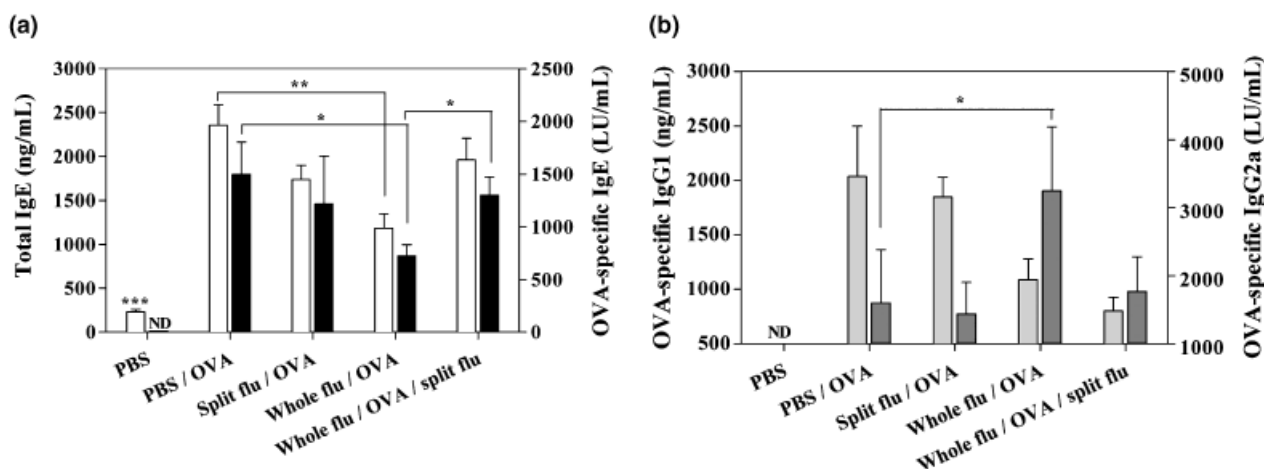*Airway inflammation.* The majority of cells recovered from BAL fluids of negative controls were differentiated

Fig. 1. Effect of influenza vaccination on allergen-induced sensitization. Mice were immunized as described in Table 1. On day 32, blood samples were collected, and serum levels of (a) total IgE (white bars) and OVA-specific IgE (black bars), (b) OVA-specific IgG1 (light grey bars) and IgG2a (dark grey bars) were measured by means of ELISA (one-way ANOVA with Tukey's multiple comparison post test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$). ND, below quantification limit; PBS, phosphate-buffered saline; OVA, ovalbumin.
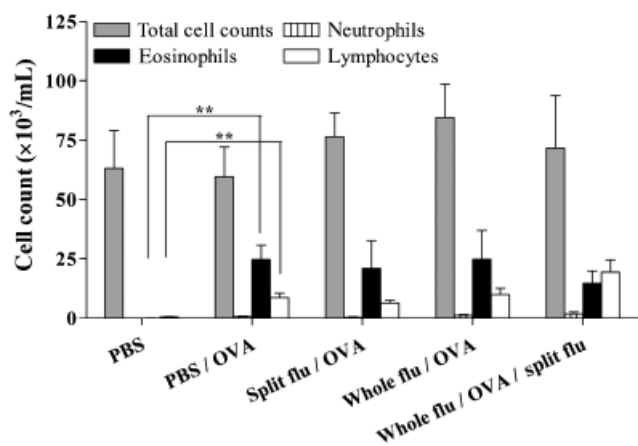

Fig. 2. Effect of influenza vaccine on allergen-mediated AI. Mice were immunized as described in Table 1. AI was examined by means of differentiation of cells recovered by bronchoalveolar lavage (one-way ANOVA with Tukey's multiple comparison post test: **$P < 0.01$). PBS, phosphate-buffered saline; OVA, ovalbumin; AI, airway inflammation.

as macrophages. OVA airway challenges of sensitized mice caused allergen-induced AI shown by a highly significant influx of lymphocytes and eosinophils into the lungs (Figs 2 and 3). Influenza vaccination, using split or whole virion vaccine, before OVA sensitization did not significantly alter the cellular pattern of BAL fluids compared with positive control (PBS/OVA), and neither did the addition of a split flu boost before OVA challenge (whole flu/OVA/split flu) (Figs 2 and 3). Treatment with influenza vaccine induced a non-significant increase in total BAL fluid cells, which was mainly caused by an increased influx of macrophages.

Airway reactivity. OVA sensitization and airway challenges caused increased *in vivo* AR in response to unspecific airway provocation with MCh as shown by significantly increased Penh values compared with negative controls (Fig. 4). Maximal Penh values of mice receiving vaccination with whole virion before first sensitization (whole flu/OVA) were significantly decreased when compared with positive control (PBS/OVA), and the resulting values were equivalent to those of negative controls (PBS). Similarly, vaccination with the split form of influenza before sensitization (split flu/OVA) also showed reduced levels of *in vivo* AR after allergen airway challenges, although to a non-significant extent, in comparison with positive control (PBS/OVA). In the group receiving additional vaccination before allergen airway challenges (whole flu/OVA/split flu), the inhibitory effect of the priming on *in vivo* AR was abrogated, resulting in maximal Penh values similar to those in positive control (PBS/OVA) (Fig. 4).

Effect of influenza vaccination on unspecific immune responses

Proliferative responses. All OVA-sensitized groups showed significantly enhanced proliferative responses of spleen MNCs following mitogenic stimulation with ConA, when compared with negative controls (Table 2). Flu vaccine treatment did not alter unspecific proliferative responses of this kind.

In vivo *cytokine production.* Vaccination with whole flu vaccine before allergen sensitization (whole flu/OVA) induced a highly significant reduction of *in vitro* IL-5
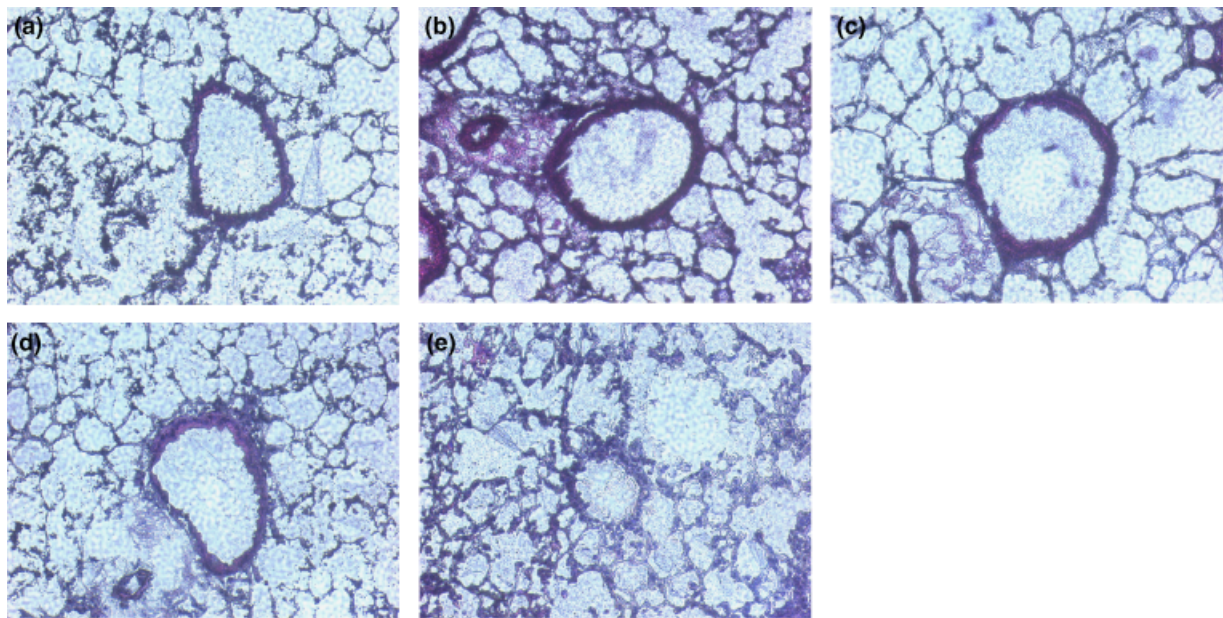
Fig. 3. Effect of influenza vaccination on allergen-mediated cellular accumulation in lung tissues. Mice were immunized as described in Table 1. On day 32, lungs were removed for histology. (a) Negative controls (PBS), (b) positive controls (PBS/OVA), (c) split flu/OVA, (d) whole flu/OVA, (e) whole flu/OVA/split flu/OVA. PBS, phosphate-buffered saline; OVA, ovalbumin.
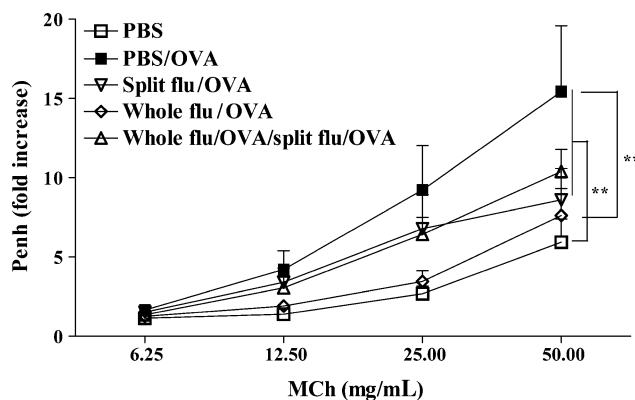

Fig. 4. Effect of influenza vaccine on allergen-mediated AR. Mice were immunized as described in Table 1. *In vivo* AR was determined by whole-body plethysmography in response to increasing doses of aerosolized MCh. Data are expressed as increases of Penh values for each concentration of MCh relative to baseline Penh values (one-way ANOVA with Tukey's multiple comparison post test on maximal Penh values: $**P < 0.01$). PBS, phosphate-buffered saline; OVA, ovalbumin; AR, airway reactivity; MCh, methacholine.

production by PBLN cells stimulated with PMA-Ionomycin, compared with positive control (PBS/OVA) (Table 3). In contrast, unspecific IL-5 production by PBLN cells was sharply increased in the mice receiving additional vaccination during sensitization (whole flu/OVA/split flu), resulting in IL-5 levels equivalent to positive controls (PBS/OVA) and significantly higher than IL-5 levels in mice vaccinated only before sensitization (whole flu/OVA).

Effect of influenza vaccination on allergen-specific immune responses

Proliferative responses. After OVA sensitization, the proliferative responses of spleen MNCs after stimulation with OVA *in vitro* were significantly increased compared with negative control (PBS) (Table 2). Vaccination with whole flu before allergen sensitization significantly suppressed allergen-specific immune responses of spleen MNCs compared with positive control (PBS/OVA) and resulted in responses similar to those in negative control (PBS) (Table 2). This suppressive effect on allergen-specific immune responses was completely abrogated when vaccination was additionally performed during sensitization right before allergen airway challenges (whole flu/OVA/split flu), resulting in levels equivalent to those in positive control animals. Even unstimulated spleen MNCs from this group (whole flu/OVA/split flu) showed significantly enhanced proliferation compared with all other groups.

In vitro cytokine production. Both vaccination regimens using whole virion (whole flu/OVA and whole flu/OVA/split flu) resulted in slightly increased *in vitro* production of the Th1 cytokine IFN-γ by spleen MNCs in response to OVA, whereas systemic production of IL-5 and IL-10 was not significantly affected by vaccination (Table 2). In contrast, vaccination with whole flu before OVA sensitization not only increased *in vitro* production of IFN-γ but also markedly reduced the production of the Th2 cytokine IL-5 and of the regulatory cytokine IL-10 by the local

Table 2. Effect of influenza vaccination on *in vitro* immune responses of spleen MNCs

	^{3}H-thymidine incorporation (c.p.m.)			OVA *in vitro*		
Treatment	Medium	OVA	ConA	IFN-γ (pg/mL)	IL-5 (ng/mL)	IL-10 (ng/mL)
PBS	680 ± 126	908 ± 367	100 256 ± 15293[c]			
PBS/OVA	356 ± 54	7053 ± 933[b]	161 296 ± 2888	14.4 ± 4.1	2.81 ± 0.60	7.55 ± 1.05
Whole flu/OVA	507 ± 216	3897 ± 1842	150 368 ± 18554	25.9 ± 4.8	3.25 ± 0.67	8.93 ± 1.17
Whole flu/OVA/split flu	3522 ± 411[a]	8404 ± 1090[b]	147 461 ± 6441	26.1 ± 11.0	2.76 ± 0.89	5.31 ± 1.30

Mice were immunized as described in Table 1. On day 32, spleen MNCs were isolated and cultured with medium, OVA (50 μg/mL) or ConA (2.5 μg/mL). The proliferative responses of cultured spleen MNCs were determined by ^{3}H-thymidine incorporation (0.5 μCi/200 μL). Levels of cytokines were assessed in cell culture supernatants of spleen MNCs by ELISA.
[a]$P < 0.01$ vs. other groups, [b]$P < 0.05$ vs. PBS and Whole flu/OVA, [c]$P < 0.05$ vs. other groups.
OVA, ovalbumin; PBS, phosphate-buffered saline; MNC, mononuclear cell; ConA, Concanavalin A; c.p.m., counts per minute.

Table 3. Effect of influenza vaccination on *in vitro* cytokine production by PBLN cells

	OVA *in vitro*			PMA+Ionomycin *in vitro*
Treatment	IFN-γ (pg/mL)	IL-5 (ng/mL)	IL-10 (ng/mL)	IL-5 (pg/mL)
PBS/OVA	25.9 ± 9.2	17.22 ± 3.20	21.62 ± 3.27	1.98 ± 0.39
Whole flu/OVA	51.5 ± 6.3[a]	8.70 ± 3.75[a]	12.13 ± 2.83[a]	0.30 ± 0.11[a]
Whole flu/OVA/split flu	39.7 ± 13.0	18.05 ± 0.65	24.50 ± 3.75	3.17 ± 0.32

Mice were immunized as described in Table 1. On day 32, PBLN cells were isolated and cultured with OVA, (50 μg/mL) or PMA+Ionomycin (PMA; 10 ng/mL, Ionomycin; 1 μg/mL). Levels of cytokines were assessed in cell culture supernatants of PBLN by ELISA.
[a]$P < 0.01$ vs. other groups.
OVA, ovalbumin; PBS, phosphate-buffered saline; PBLN, peribronchial lymph node; PMA, phorbol-12-myristate-13-acetate.

draining lymph nodes (PBLN) after restimulation with OVA (Table 3). Thus, whole flu vaccination generated a switch towards a predominant Th1-immune response in the local compartment of the allergic airway response. The addition of a split flu boost before OVA challenge attenuated this immune-modulating effect of whole flu vaccination, as indicated by decreased IFN-γ levels, increased IL-5 levels and up-regulation of IL-10 production in these animals (whole flu/OVA/split flu) compared with the latter group (whole flu/OVA) (Table 3).

Discussion

Until now, experimental studies of the link between influenza virus and allergic airway diseases have focused on the effect of infection with live wild-type virus on the inception of atopic asthma. One of the main outcomes of these animal studies was that, depending on the time-point of infection, influenza either prevented or exacerbated the allergen-induced sensitization [4, 16]. In the present study, we showed for the first time that intranasal instillation of whole influenza vaccine before allergen sensitization inhibited subsequent development of allergen-mediated sensitization and airway hyper-reactivity (AHR), both associated with a local shift from a predominant Th2- towards a Th1-immune response in the local compartment of the allergic airway reaction.

The cardinal features of allergic airway disease – allergen-specific IgE formation and the development of AHR and eosinophilic AI – were shown to depend on Th2-type cytokines [17–20]. They can be inhibited by application of Th1-cytokines such as IFN-γ, IL-12 and IL-18 as well as by tolerance induction via regulatory T cells [10, 11, 21]. In particular, previous studies have shown that influenza infection before allergen sensitization induced local IFN-γ production, which blocked the development of allergen-induced AHR and led to reduced activation of allergen-specific Th2 cells [10, 22]. It is therefore likely that the pulmonary Th1 shift induced by intranasal instillation of whole influenza vaccine was responsible for the observed inhibition of AHR in our model. Although the allergen-specific Th1 shift was predominant in the local pulmonary compartment, whole flu vaccination also induced systemic immune modulation such as impaired allergen-specific proliferation of spleen MNCs, increased allergen-specific IgG2a and decreased IgG1 as well as allergen-specific IgE serum levels. It is most likely that the underlying mechanism is linked to increased IFN-γ and Th1 induction in response to flu vaccination, thus inhibiting the clonal expansion of Th2 cells and suppressing IgE production during primary sensitization [23]. Of specific interest was the notion that whole influenza vaccination also decreased the allergen-specific *in vitro* production of IL-10 by PBLN cells,

which allowed us to exclude mucosal tolerance induction as the explanation for the observed immune suppression [21].

Interestingly, the i.n. instillation of whole virion vaccine before OVA sensitization showed a stronger preventive effect than the split virion influenza vaccine. Differences in the immune response induced by the two vaccines following intramuscular administration have been addressed by several groups [24–26], demonstrating that whole influenza vaccination was more immunogenic, more reactogenic and induced a strong Th1 immune response, while split influenza vaccination rather elicited a more mixed Th1/Th2 profile. This confirms our results as it indicates the important role of Th1 induction for the preventive effect of whole flu priming. The exact mechanism underlying the differences in the immune modulation by the two vaccination compounds remains unknown, but may be explained by the differences in their respective composition and structure, as split vaccines lack the viral lipid envelope and contain less viral RNA than whole influenza vaccines. As single-stranded RNA has been found to stimulate Th1 responses by activation of toll-like receptors 7 and 8 located on the endosomal membranes [27, 28], the increased content of this material in whole flu vaccine compared with split vaccine may very well explain the stronger Th1 response observed after vaccination with the former compound. The presence of the viral lipids (such as cholesterol, sphingomyelin and phosphatidylcholine [29]), and of the viral envelope as a whole, could further increase the Th1 bias by facilitating entry of RNA into the target cells. In support of this thinking, it has been demonstrated that egg-prepared liposomes are able to facilitate entry of exogenous antigens into the Th1 pathway in BALB/c mice [30].

In contrast to the effects on allergen-mediated sensitization and AHR, i.n. whole virion influenza vaccination did not reduce the infiltration of eosinophils into the airways, despite a sharp reduction of *in vitro* IL-5 produced by allergen-stimulated PBLN cells. This discrepancy between airway eosinophilia on the one hand and the other characteristic features of allergic asthma (namely AHR) on the other has already been reported several times [31], and suggests that eosinophilia does not solely rely on IgE or local IL-5 production. Other mediators may take over the job to attract and activate eosinophils in the airways in the absence of IgE and/or IL-5. Such a candidate is eotaxin (CCL11), a potent and selective chemokine that was found to be an important factor in regulating eosinophil migration [32]. As the expression of eotaxin by lung epithelial cells was shown to be stimulated by IFN-γ [33], this may be the missing link explaining the increased BAL eosinophil numbers in the whole flu-vaccinated allergen-sensitized and challenged mice.

The addition of a split influenza boost preceding the OVA challenges (whole flu/OVA/split flu) limited the preventive effect of whole flu priming on OVA sensitization. While the addition of the split flu boost did not affect lung eosinophilia, we did observe an increase in OVA-specific IgE levels and a non-significant aggravation of AHR. These observations were correlated with decreased levels of IFN-γ and increased levels of allergen-specific IL-5 and IL-10 production by local draining lymph node (PBLN) cells. This emphasizes the importance of the Th1 shift in the preventive effect of whole flu priming, which was weakened by the subsequent addition of a split flu boost, thus restoring the asthmatic phenotype. Moreover, the split flu boost unspecifically reactivated central B and T lymphocytes as witnessed by the increased proliferation of medium-stimulated spleen MNCs. Altogether, these results showed an important role of the temporal kinetics as the sequence of vaccinations and allergen sensitization was crucial for the inhibitory effects.

Currently available evidence suggests that influenza A infection early in life may prevent experimental allergic airway disease through increased IFN-γ production [10, 11]. We demonstrated here that inactivated virus vaccines, which are currently licensed only for intramuscular (i.m.) application, also maintained, to various extents, the capacity to protect from allergen-induced airway disease when administered i.n. This protective effect was most marked with whole influenza vaccine and associated with its Th1-inducting potential. When administered i.m., whole virus influenza vaccine is known to be more immunogenic and reactogenic (specifically, more pyrogenic) as compared with subunit/split virus vaccine, both in mice and in human subjects [34–36]. Owing to this increased reactogenicity, the use of whole virus vaccine in children has been discontinued in many countries. However, in the current effort to develop effective and immunogenic vaccines against avian influenza viruses of pandemic potential, whole virus influenza vaccines are being re-discovered [37]. In the present study, we illustrated that these vaccines also demonstrate a very promising potential for the primary prevention of allergic diseases such as asthma. Our data suggest that inactivated whole virion influenza vaccines might be a promising new approach that could be tested in humans as a safe means to reproduce the protective effect of early childhood infections by using a single intranasal immunization during the first year of life. As the exact timing of allergen sensitization is still a matter of debate, we also recommend further investigations of the effect of intranasal whole influenza vaccination in already sensitized individuals. Moreover, the protective efficacy of whole virus inactivated influenza vaccine with regard to prevention of hyper-reactive airway disease will need to be compared with that of live-attenuated intranasal influenza vaccine.

Acknowledgements

References

1 Trujillo C, Erb KJ. Inhibition of allergic disorders by infection with bacteria or the exposure to bacterial products. *Int J Med Microbiol* 2003; **293**:123–31.

2 Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 2003; **3**:733–44.

3 Illi S, von Mutius E, Lau S *et al.* Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. *BMJ* 2001; **322**:390–5.

4 Kamradt T, Goggel R, Erb KJ. Induction, exacerbation and inhibition of allergic and autoimmune diseases by infection. *Trends Immunol* 2005; **26**:260–7.

5 Gerhold K, Bluemchen K, Franke A, Stock P, Hamelmann E. Exposure to endotoxin and allergen in early life and its effect on allergen sensitization in mice. *J Allergy Clin Immunol* 2003; **112**:389–96.

6 Gruber C, Gerhold K, von Stuckrad SL *et al.* Common vaccine antigens inhibit allergen-induced sensitization and airway hyperresponsiveness in a murine model. *Allergy* 2006; **61**:820–7.

7 Kurukulaaratchy RJ, Matthews S, Holgate ST, Arshad SH. Predicting persistent disease among children who wheeze during early life. *Eur Respir J* 2003; **22**:767–71.

8 Parkin PC, Taylor CY, Petric M, Schuh S, Goldbach M, Ipp M. Controlled study of respiratory viruses and wheezing. *Arch Dis Child* 2002; **87**:221–2.

9 Message SD, Johnston SL. Viruses in asthma. *Br Med Bull* 2002; **61**:29–43.

10 Marsland BJ, Harris NL, Camberis M, Kopf M, Hook SM, Le Gros G. Bystander suppression of allergic airway inflammation by lung resident memory CD8+ T cells. *Proc Natl Acad Sci USA* 2004; **101**:6116–21.

11 Wohlleben G, Muller J, Tatsch U *et al.* Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J Immunol* 2003; **170**:4601–11.

12 Brady MI, Furminger IG. A surface antigen influenza vaccine. 1. Purification of haemagglutinin and neuraminidase proteins. *J Hyg (London)* 1976; **77**:161–72.

13 Hamelmann E, Oshiba A, Loader J *et al.* Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 1997; **155**:819–25.

14 Hamelmann E, Schwarze J, Takeda K *et al.* Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997; **156**:766–75.

15 Gerhold K, Blumchen K, Bock A *et al.* Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 2002; **110**:110–6.

16 Yamamoto N, Suzuki S, Suzuki Y *et al.* Immune response induced by airway sensitization after influenza A virus infection depends on timing of antigen exposure in mice. *J Virol* 2001; **75**:499–505.

17 Taube C, Dakhama A, Gelfand EW. Insights into the pathogenesis of asthma utilizing murine models. *Int Arch Allergy Immunol* 2004; **135**:173–86.

18 Mould AW, Ramsay AJ, Matthaei KI, Young IG, Rothenberg ME, Foster PS. The effect of IL-5 and eotaxin expression in the lung on eosinophil trafficking and degranulation and the induction of bronchial hyperreactivity. *J Immunol* 2000; **164**:2142–50.

19 Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med* 1997; **185**:785–90.

20 Shi H, Qin S, Huang G *et al.* Infiltration of eosinophils into the asthmatic airways caused by interleukin 5. *Am J Respir Cell Mol Biol* 1997; **16**:220–4.

21 Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005; **116**:961–8.

22 Tsitoura DC, Kim S, Dabbagh K, Berry G, Lewis DB, Umetsu DT. Respiratory infection with influenza A virus interferes with the induction of tolerance to aeroallergens. *J Immunol* 2000; **165**:3484–91.

23 Pene J, Rousset F, Briere F *et al.* IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN-gamma. *J Immunol* 1988; **141**:1218–24.

24 Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. *Scand J Immunol* 2005; **62**:36–44.

25 Cox RJ, Hovden AO, Brokstad KA, Szyszko E, Madhun AS, Haaheim LR. The humoral immune response and protective efficacy of vaccination with inactivated split and whole influenza virus vaccines in BALB/c mice. *Vaccine* 2006; **24**:6585–7.

26 Gross PA, Ennis FA, Gaerlan PF, Denson LJ, Denning CR, Schiffman D. A controlled double-blind comparison of reactogenicity, immunogenicity, and protective efficacy of whole-virus and split-product influenza vaccines in children. *J Infect Dis* 1977; **136**:623–32.

27 Lund JM, Alexopoulou L, Sato A *et al.* Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci USA* 2004; **101**:5598–603.

28 van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. *Trends Immunol* 2006; **27**:49–55.

29 Takeda M, Leser GP, Russell CJ, Lamb RA. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc Natl Acad Sci USA* 2003; **100**:14610–7.

30 Jaafari MR, Ghafarian A, Farrokh-Gisour A *et al.* Immune response and protection assay of recombinant major surface glycoprotein of *Leishmania* (rgp63) reconstituted with liposomes in BALB/c mice. *Vaccine* 2006; **24**:5708–17.

31 Cho YS, Kwon B, Lee TH *et al.* 4-1BB stimulation inhibits allergen-specific immunoglobulin E production and airway hyper-reactivity but partially suppresses bronchial eosinophilic inflammation in a mouse asthma model. *Clin Exp Allergy* 2006; **36**:377–85.

32 Mattes J, Yang M, Mahalingam S *et al.* Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp Med* 2002; **195**:1433–44.

33 Lilly CM, Nakamura H, Kesselman H *et al.* Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J Clin Invest* 1997; **99**: 1767–73.

34 Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand J Immunol* 2004; **59**:1–15.

35 Wright PF, Dolin R, La Montagne JR. From the National Institute of Allergy, infectious diseases of the National Institutes of Health, the Center for Disease Control and the Bureau of Biologics of the Food and Drug Administration. Summary of clinical trials of influenza vaccines – II. *J Infect Dis* 1976; **134**:633–8.

36 Bernstein DI, Zahradnik JM, DeAngelis CJ, Cherry JD. Clinical reactions and serologic responses after vaccination with whole-virus or split-virus influenza vaccines in children aged 6 to 36 months. *Pediatrics* 1982; **69**:404–8.

37 Lin J, Zhang J, Dong X *et al.* Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet* 2006; **368**:991–7.