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

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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 0 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.

Keywords asthma, cytokines, influenza vaccine, mice, ovalbumin

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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 countering allergies-inducing 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-mediated sensitization and challenge, mice were primed with vaccines as described above and additionally receiving whole influenza virus and found no induction of AI by the inactivated influenza virus strains [5 μg hemagglutinin (HA)/strain, A/Wyoming/3/2003, A/New Caledonia/20/99, B/Yangsus/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/Yangsus/10/2003, GlaxoSmithKline Biologicals) (whole flu/OVA, n = 12) (Table 1).

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/Yangsus/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/Yangsus/10/2003, GlaxoSmithKline Biologicals) (whole flu/OVA, 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 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 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 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).

**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.

**Table 1. Sensitization and immunization protocol**

<table>
<thead>
<tr>
<th>Group</th>
<th>Flu priming (day –1)</th>
<th>OVA sensitization (days 1, 14)</th>
<th>Flu boost (day 27)</th>
<th>OVA challenge (days 28–30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control: PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Positive control: PBS/OVA</td>
<td>PBS</td>
<td>OVA</td>
<td>PBS</td>
<td>OVA</td>
</tr>
<tr>
<td>Split flu/OVA</td>
<td>Split flu</td>
<td>OVA</td>
<td>PBS</td>
<td>OVA</td>
</tr>
<tr>
<td>Whole flu/OVA</td>
<td>Whole flu</td>
<td>OVA</td>
<td>PBS</td>
<td>OVA</td>
</tr>
<tr>
<td>Whole flu/OVA/split flu</td>
<td>Whole flu</td>
<td>OVA</td>
<td>Split flu</td>
<td>OVA</td>
</tr>
</tbody>
</table>

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.

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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 (Reichert-Jung, Nü-ßloch, 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 \(-70^\circ C\). Sections (8 \( \mu \)m 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 \( \mu \)g/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 \( \mu \)g/mL) or OVA (Grade VI, 50 \( \mu \)g/mL) were determined by \(^3\)H-thymidine incorporation (0.5 \( \mu \)Ci/200 \( \mu \)L, 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 \( \mu \)g/mL, Ionomycin; 1 \( \mu \)g/mL) or OVA (50 \( \mu \)g/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-\( \gamma \) and IL-10 were assessed by Pharmingen OptEia kits (Pharmentigen, 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
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
production by PBLN cells stimulated with PMA-Ionomycin, compared with positive control (PBS/OVA) (Table 3). In contrast, un-specific 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
Whole influenza vaccination prevents allergic airway disease in mice

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>³H-thymidine incorporation (c.p.m.)</th>
<th>OVA in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>OVA</td>
</tr>
<tr>
<td>PBS</td>
<td>680 ± 126</td>
<td>908 ± 367</td>
</tr>
<tr>
<td>PBS/OVA</td>
<td>356 ± 54</td>
<td>7053 ± 933³</td>
</tr>
<tr>
<td>Whole flu/OVA</td>
<td>507 ± 216</td>
<td>3897 ± 1842</td>
</tr>
<tr>
<td>Whole flu/OVA/split flu</td>
<td>3522 ± 411³</td>
<td>8404 ± 1090³</td>
</tr>
</tbody>
</table>

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 ³H-thymidine incorporation (0.5 µCi/200 µL). Levels of cytokines were assessed in cell culture supernatants of spleen MNCs by ELISA.

³P < 0.01 vs. other groups, ⁴P < 0.05 vs. PBS and Whole flu/OVA, ⁵P < 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OVA in vitro</th>
<th>PMA + Ionomycin in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ (pg/mL)</td>
<td>IL-5 (ng/mL)</td>
</tr>
<tr>
<td>PBS/OVA</td>
<td>25.9 ± 9.2</td>
<td>17.22 ± 3.20</td>
</tr>
<tr>
<td>Whole flu/OVA</td>
<td>51.5 ± 6.3a</td>
<td>8.70 ± 3.75a</td>
</tr>
<tr>
<td>Whole flu/OVA/split flu</td>
<td>39.7 ± 13.0</td>
<td>18.05 ± 0.65</td>
</tr>
</tbody>
</table>

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.

⁴P < 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 IL-10. 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 stronger 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 IFN-\(\gamma\), demonstrated 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. 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

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