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Original Article

Immunization with live influenza viruses in an experimental model of allergic bronchial asthma: infection and vaccination

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Background Asthmatics in particular have a need for influenza vaccines because influenza infection is a frequent cause of hospitalization of patients with bronchial asthma. Currently, only inactivated influenza vaccines are recommended for influenza prevention in asthma sufferers.

Objective The aim of our study was to analyze and compare the effects of influenza infection and vaccination with live attenuated influenza vaccine (LAIV) on different phases of experimental murine allergic bronchial asthma (acute asthma and remission phase) and on subsequent exposure to allergen in sensitized animals.

Methods Ovalbumin (OVA)-specific serum IgE levels, IL-4 production by spleen and lung lymphocytes, and histological changes in the lungs of mice infected with pathogenic virus or LAIV were studied at two phases of OVA-induced bronchial asthma (acute asthma and remission).

Results Infection with pathogenic virus both in acute asthma and remission led to asthma exacerbation associated with the production of OVA-specific IgE, IL-4 and significant inflammatory infiltration in airways. Infection, even after complete virus clearance, induced the aggravation of lung inflammation and IgE production in asthmatic mice additionally exposed to OVA. Immunization with LAIV at remission did not enhance allergic inflammatory changes in the lung, OVA-specific IgE or IL-4 production. Then after additional OVA exposure, histological and immunological changes in these mice were the same as in the control group.

Conclusions Influenza infection provokes asthma exacerbation regardless of the disease phase. Immunization with LAIV during the remission phase of bronchial asthma is safe and does not interfere upon subsequent contact of asthma sufferers with allergen.

Keywords Asthma, influenza infection, live attenuated influenza vaccine.

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Introduction

Bronchial asthma is a chronic inflammatory respiratory disease, predominantly of allergic etiology, characterized by bronchial hyper-responsiveness and bronchial obstruction.[1] There are about 300 million people worldwide suffering from asthma; approximately, 10% of North America population and 2·5–10% of Europeans have asthma.[2] Epidemiological studies revealed that influenza virus infection is a frequent cause of hospitalization of patients with asthma exacerbation.[3–5] Therefore, asthmatics are in particular need of influenza vaccine prophylaxis.[6,7]

Currently, two types of influenza vaccines are being produced worldwide: various versions of the inactivated influenza vaccines (IIV) administered parenterally and live attenuated reassortant vaccines (LAIV) which are administered intranasally. In the USA, for the bronchial asthma sufferers, IIV are recommended for influenza prevention as safe low-allergenic vaccines.[8] However, some authors believe the efficacy of such vaccines to be low for asthmatics, especially for children.[9–11] The alternative to IIV are LAIV produced by genetic reassortment of an actual pathogenic strain with a cold-adapted attenuated donor virus.

There are two LAIV vaccines currently licensed for use in Russia and in USA respectively and these differ in the attenuation donor strain. Some comparative studies of IIV and American LAIV revealed the live vaccine demonstrate greater efficacy and effectiveness.[12,13] Recently, some clinical LAIV safety studies have been carried out with a limited cohort of bronchial asthma sufferers. These studies provided

evidence that this vaccine was safe for children aged above 6 and adolescents with asthma of low and medium severity[14,15] and LAIV was more effective than IIV for children with asthma.[15] However, the authors focused their attention predominantly on physiological measurements, forced expiratory volume and peak expiratory rate. It is important to understand the influence of vaccination on the immunological parameters of allergic asthma inflammation.

This work was performed to compare the allergic immunological consequences of influenza infection and LAIV vaccination induced in an experimental bronchial asthma model in mice. We studied the effects of influenza virus infection and immunization with LAIV on acute asthma and remission phases of experimental murine allergic bronchial asthma and on subsequent exposure to allergen in sensitized animals post-infection or post-vaccination.

Materials and methods

Animals

Female CBA mice 6–8 weeks of age were purchased from the State Breeding Farm "Rappolovo" (St. Petersburg, Russia) and kept in pathogen-free conditions. Animals were maintained under the approved guidelines of the Ministry of Healthcare and Social Development of the Russian Federation.

OVA-induced bronchial asthma protocols

Bronchial asthma in mice was induced by intraperitoneal/intranasal OVA-sensitization as described by Tsitoura *et al.*[16] (Figure 1). Mice were lightly anesthetized and injected i.p. with 100 μg OVA (MP Biomedicals, Inc., Aurora, OH, USA) in 2 mg aluminum hydroxide adjuvant (day 0). Ten days later, mice received 50 μg OVA in phosphate-buffered saline (PBS) i.n. on three consecutive days (days 10–12). Development of bronchial asthma was confirmed by increased levels of OVA-specific IgE as well as representative inflammatory changes in lung histology. Forty eight hours or 10 days after the last OVA inoculation (day 14 or 22) influenza infection or vaccination was performed, as described below, in the acute phase (day 14) or remission (day 22) of asthma respectively. Fourteen days later, when the virus had been completely eliminated, mice were additionally exposed to allergen (50 μg OVA i.n.) three times. Sampling of blood and organs was performed 48 hours after inoculation of viruses or subsequent OVA administration respectively.

Influenza infection and vaccination

Mice were anesthetized and inoculated i.n. with influenza virus in 50 μl of PBS. To obtain influenza infection 4·0 $1ogEID_{50}$ of influenza virus A/PR/8/34 H1N1 was administered. Our preliminary studies had shown that this dose of pathogenic virus causes severe but non-lethal infection with complete viral clearance by day 10–12 after inoculation. Mice from the LAIV group were treated with attenuated 6:2 reassortant H1N1 virus (Len/47 × PR/8) prepared by genetic reassortant between pathogenic A/PR/8/34 (H1N1) virus and the cold-adapted A/Leningrad/134/47/57 (H2N2) attenuation donor. Len/47 × PR/8 inherited two genes encoding surface antigens from the pathogenic virus and six internal genes from the

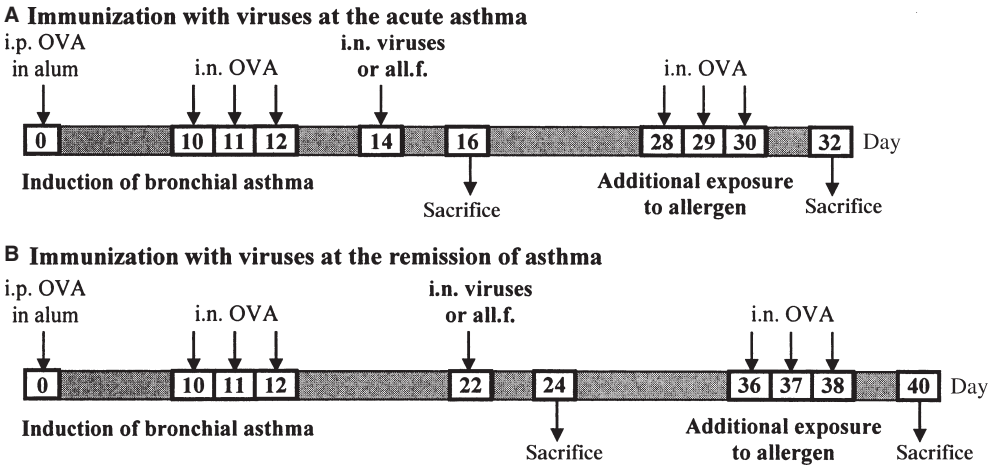

Figure 1. Experimental protocols. For induction of bronchial asthma mice were sensitized on day 0 with i.p. OVA in alum adjuvant and then 10 days later exposed to i.n. OVA on three successive days. After 48 hours (A – during acute asthma) or in 10 days (B – during asthma remission) after the final i.n. OVA inoculation mice were immunized with pathogenic or attenuated reassortant influenza viruses or with the allantoic fluid as control. Fourteen days after the virus or allantoic fluid. inoculation mice were additionally exposed to i.n. OVA three times on successive days. The sampling of blood and organs was performed 48 hours after virus or subsequent OVA administration.

attenuated donor strain. Such a reassortment scheme and A/Leningrad/134/47/57 (H2N2) attenuation donor are used in preparing vaccine strains for the Russian LAIV.[17] The Len/47 × PR/8 attenuated reassortant had *ca*- (cold-adapted) and *ts*- (temperature sensitive) phenotype multiplying successfully at +26°C but not being able to reproduce at +38°C. Len/47 × PR/8 virus was administered at 6·0 $1\log EID_{50}$ as a vaccine dose as used in the Russian LAIV. The control animals received virus-free allantoic fluid (all.f.) diluted 1:100 in PBS.

The replication properties of Len/47 × PR/8 in the upper and lower respiratory tract of CBA mice were tested in our preliminary studies. The virus titers were determined by titration in chicken embryos and calculating the 50% endpoint by the method of Reed and Muench.[18] The virus reproduced well in the upper respiratory tract (virus titers in the nasopharynx tissue homogenate were 3·0–4·2 $1gEID_{50}$/0·1 ml) and poorly in lungs (1·0–1·7 $1gEID_{50}$/0·1 ml).

Hemagglutination inhibition activity

The hemagglutination inhibition (HAI) assay was performed using a standard procedure[19] with a working stock of viral antigen containing four hemagglutination units (HAU) per well. To inactivate non-specific inhibitors, sera were heated in a 56°C water bath for 30 min and treated with a 1% chicken red blood cells suspension for 30 min at room temperature. The HAI titers were determined by the reciprocal dilution of the last row which contained non-agglutinated chicken erythrocytes.

Total serum IgE

Mice were bled at the time of killing and total serum IgE was analyzed by ELISA using standard commercial kits (BD Biosciences Pharmingen, San Diego, CA, USA).

Virus-specific serum IgG

Virus-specific IgG titers were measured using a virus-specific ELISA method developed previously.[20] Influenza A/PR/8/34 (H1N1) virus antigens purified by ultracentrifugation were adsorbed to ELISA plates at 16 HAU/well and incubated overnight at 4°C. After washing and blocking, serial dilutions of sera were added for 2 hours. Then plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-IgG (Cappel ICN Inc., Aurora, OH, USA), washed and developed by adding *o*-phenylenediamine substrate. The optical density (OD) was determined using a microplate autoreader (Bio-Tec Instruments, Inc., Winooski, Vermont, USA) at 490 nm. Virus-specific IgG titers were defined as the last serum dilution at which the OD was at least twofold higher than the OD of control data (all the ingredients of the reaction except the serum sample). The concentrations of virus-specific IgG were determined by comparison with a standard serum obtained from naïve animals (as a negative control).

OVA-specific serum IgE

Measurement of OVA-specific serum IgE was performed using an OVA-specific ELISA. Plates were coated with 10 μg/ml OVA overnight at 4°C, then washed and blocked. Serum samples were added for 2 hours. at 37°C. After washing, plates were overlaid with HRP-conjugated goat anti-IgE (Bethyl, Inc., Montgomery, TX, USA), reacted for 1·5 hours. at 37°C, and then incubated with 3,3′,5,5′-tetramethylbenzidine (TMB; BD Biosciences Pharmingen) for 30 minutes at room temperature. A colorimetric assay of the plates was performed at 450 and 570 nm. The concentrations of specific antibodies were determined by comparison with a standard curve obtained from serial dilutions of standard serum. OVA-specific IgE standard serum was obtained from animals hyperimmunized with OVA in alum.

Spleen and lung lymphocytes separation

Spleen lymphocytes were harvested by tissue homogenizing and suspending lymphocytes in an erythrocyte-lysing ACK buffer. Separation of lung lymphocytes was performed by the method of Saxena *et al.*[21]. Lungs were removed, homogenized in 5 ml complete RPMI-1640 (Biolot Co, St. Petersburg, Russia) and incubated with TES-calcium buffer (pH 7·5) containing 1 mg/ml collagenase (MP Biomedicals, Inc.) for 0·5 hours. at 37°C, then washed twice with PBS. Lymphocytes were separated by density-gradient centrifugation on 70% Percoll (MP Biomedicals, Inc.). Cells were taken from the interface, washed twice with PBS and resuspended in complete RPMI-1640 with 10% fetal bovine serum (FBS) (Biolot Co, Russia).

In vitro proliferation of spleen and lung lymphocytes

The proliferative activity of spleen and lung lymphocytes was evaluated by the Mosmann method.[22] Cells were resuspended in complete RPMI-1640 with 10% FBS and cultured (2×10^6 cells/well) with or without additional stimulants: A/PR/8/34 (H1N1) influenza virus, inactivated for 30 minutes at +56°C, at a concentration 20 HAU/well or 100 μg/ml OVA. After 72 hours. 10 μl 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St Louis, MO, USA) was added to each well at a final concentration of 5 μg/ml and plates were incubated for 4 hours at 37°C. Following incubation, 100 μl of extracting solution (0,04N HC1 in isopropanol) was added to each well. A colorimetric assay of the plates was performed at 570 nm. The lymphocyte stimulation index was defined as the OD ratio of samples stimulated by virus or OVA compared to non-stimulated samples.

IL-4 levels

IL-4 levels were measured in supernatants of 3-day spleen and lung lymphocytes cultures stimulated *in vitro* with 100 μg/ml OVA. Cells cultured without OVA, failed to

produce IL-4. Assessment of IL-4 production was performed using standard commercial ELISA kits for testing mouse cytokines (BD Biosciences Pharmingen).

Histology of the lung

Lungs were removed, fixed in 10% buffered formaldehyde solution, routinely processed, and embedded in paraffin wax. Five-micrometer sections were prepared and stained with hematoxylin and eosin. The specimens were screened and photographed in a Zeiss Axiomat light microscope system (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical analysis

All data are expressed as the mean ± SD. Statistical significance was analyzed by Student's *t*-test. A *P*-value of <0·05 was considered significant.

Results

Two distinct phases of bronchial asthma

The first step of our study was to analyze the course of OVA-induced bronchial asthma in mice in order to choose the critical points for influenza infection or vaccination. For this purpose, mice were sensitized with i.p. OVA in alum adjuvant, and 10 days later, exposed to i.n. OVA three times (see Figure 1). Allergic inflammation parameters were measured on days 14 and 22 of the experiment (48 hours and 10 days after final i.n. OVA inoculation respectively). Non-sensitized mice receiving PBS instead of OVA were used as control. As expected, the total serum IgE levels in the sensitized mice at both time points were significantly higher than in control non-sensitized animals (Figure 2A). Concentrations of total IgE on day 14 and day 22 did not differ significantly. At the same time, differences were observed in OVA-specific serum IgE production. At 48 hours after the final OVA inoculation, a significant increase of OVA-specific IgE-antibody production was noted. However, on the10th day after asthma induction, OVA-specific IgE levels in serum decreased 1·5 times in comparison with the parameters measured on the 14th day. In control mice which received PBS instead of OVA, IgE specific to OVA was not found.

Lung histology of OVA-sensitized mice revealed decreased inflammation 10 days after final i.n. OVA

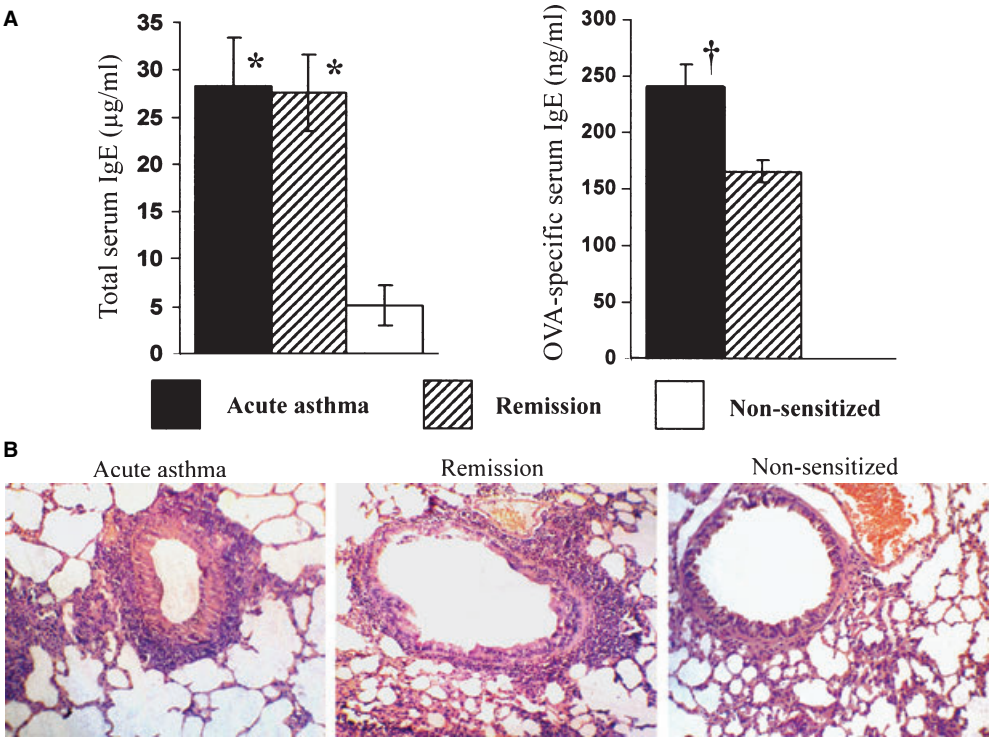

Figure 2. Description of two phases of bronchial asthma. (A) Total and OVA-specific serum IgE levels in mice at acute asthma and at remission. In non-sensitized mice OVA-specific IgE levels were less than the response limit of the ELISA (<2·00 ng/ml). **P* < 0·1 compared with the non-sensitized group, †*P* < 0·5 compared with the asthma remission group. (B) Lung histology of mice. Lungs were stained with hematoxylin and eosin (×100). Forty eight hours after the final i.n. OVA inoculation (acute asthma) obstructive emphysema and marked acute inflammation with dense peribronchial and perivascular infiltrates consisting of neutrophils and eosinophils were observed. Ten days after final intranasal OVA inoculation (remission) the acute phase of inflammation moved to a productive one characterized by macrophage and lymphocyte infiltrates. All the above mentioned pathomorphological changes are not found in the lungs of non-sensitized mice.

exposure (Figure 2B). Forty eight hours after the final i.n. OVA inoculation marked acute inflammatory processes with dense peribronchial and perivascular infiltrates, consisting of neutrophils and eosinophils, and obstructive emphysema was observed in the lungs. Ten days after the final intranasal OVA inoculation a shift from the acute inflammatory phase to the productive one was noted; lymphocytes and macrophages predominated among the cells of peribronchial infiltration, but the local sites of emphysema were maintained.

These data together have confirmed that OVA-induced bronchial asthma in mice has two distinct phases of allergic inflammation within the studied time period, the acute phase (48 hours. after the last i.n. OVA inoculation) and remission (10 days after last i.n. OVA exposure).

Virus-specific immune response

To confirm that immunity to pathogenic and vaccine influenza viruses in bronchial asthma developed fully, the virus-specific humoral and cellular immune responses were examined in infected and vaccinated groups of asthmatic mice 17 days after administration of viruses (data not shown).

At both the acute phase of asthma and at asthma remission influenza infection led to a consistent increase of virus-specific serum IgG levels and spleen lymphocyte proliferation in comparison with control animals receiving allantoic fluid instead of viruses. HAI titers were >1:40 both in mice infected at acute asthma and at remission.

Immunization with attenuated influenza virus also resulted in significant virus-specific immune response in acute asthma, and similar results were obtained in the asthma remission phase. In both cases the HAI titers were >1:40 in 100% of vaccinated mice but were 1 log less than in mice infected with the pathogenic virus.

In general, these findings demonstrated that humoral and cellular immune responses to infection and vaccination developed during both acute asthma and asthma remission.

OVA-specific IgE production

Figure 3 demonstrates OVA-specific serum IgE levels after administration of the viruses, and also after subsequent additional exposure to i.n. OVA. It should be noted that after additional administration of the allergen (OVA) to control mice there was a significant increase of OVA-specific IgE production. This provided evidence of long-term

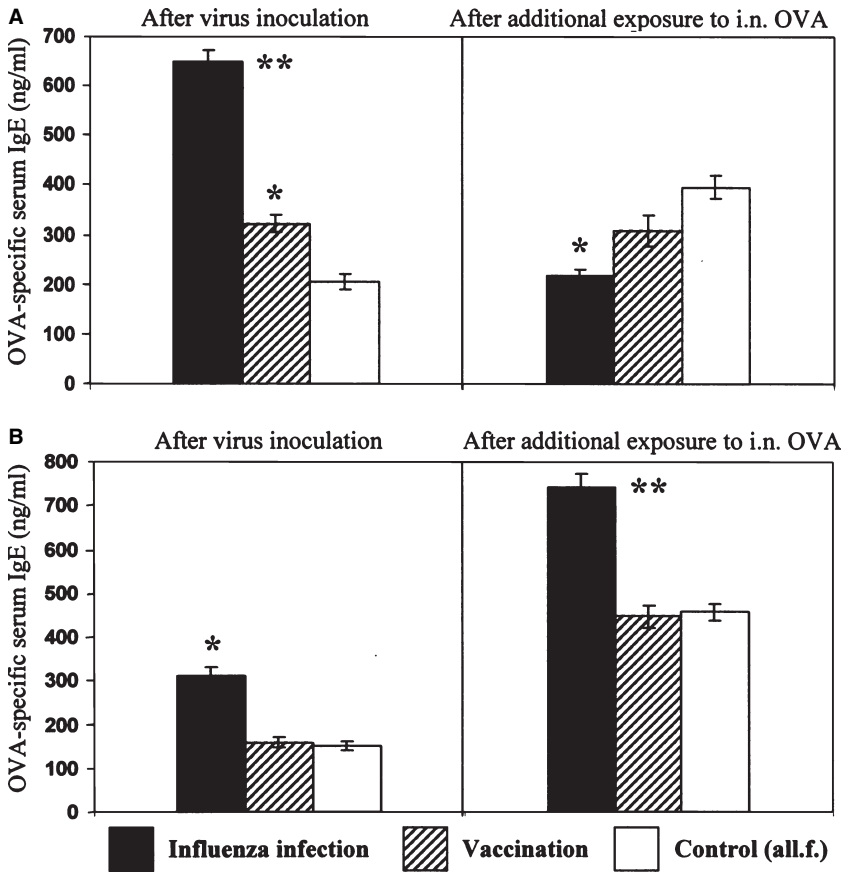

Figure 3. OVA-specific IgE production in mice surviving influenza infection or vaccination at acute asthma or at asthma remission phases. At the acute asthma (A) or at the asthma remission phase (B) mice were immunized with: A/PR/8/34 influenza virus (infection), Len/47 × PR/8 attenuated reassortant strain (vaccination), or allantoic fluid.(control). Fourteen days after virus administration mice were additionally exposed to i.n. OVA three times 1 day apart. *$P < 0{\cdot}5$, **$P < 0{\cdot}1$ compared with the control group.

(more than 16 days) maintenance of allergic reactivity to OVA in sensitized animals (see Figure 1).

Acute asthma

Influenza infection led to considerable elevation of OVA-specific IgE production over the control group (Figure 3A). However, subsequent additional exposure to allergen did not result in an increase in IgE level. Moreover, a consistent decrease of these antibodies was found in comparison with IgE levels in control group.

Immunization with attenuated influenza virus also provided a significant increase of allergen-specific IgE levels, although IgE augmentation in this case was considerably less than in infected mice. Additional allergen administration did not influence OVA-specific IgE production in asthmatic mice immunized with attenuated reassortant virus.

Asthma remission phase

During the asthma remission phase only influenza infection caused a significant increase in OVA-specific IgE levels both after virus administration and subsequent additional allergen exposure (Figure 3B). Furthermore, the peak of IgE production was observed exactly after the additional i.n. OVA administration.

In contrast to the infection group, OVA-specific IgE production in mice vaccinated during remission of asthma did not differ from that of control animals.

IL-4 production

IL-4 levels were measured in cultures of spleen and lung lymphocytes separated from asthmatic mice exposed to influenza infection or vaccination and additionally treated with OVA (day 32 or 40 - see Figure 1).

Acute asthma

In spleen lymphocytes of mice infected with pathogenic influenza virus, a decrease in IL-4 levels was observed in comparison with the control group (Figure 4A). However, in lungs, influenza infection provoked a significant increase in IL-4 levels.

IL-4 production in mice immunized with attenuated influenza virus was also lower than that in the control group both in spleen and lung lymphocyte cultures.

Asthma remission phase

Only influenza infection led to significant increase in IL-4 production both in spleen and lung lymphocyte cultures (Figure 4B). Results obtained in mice immunized with the

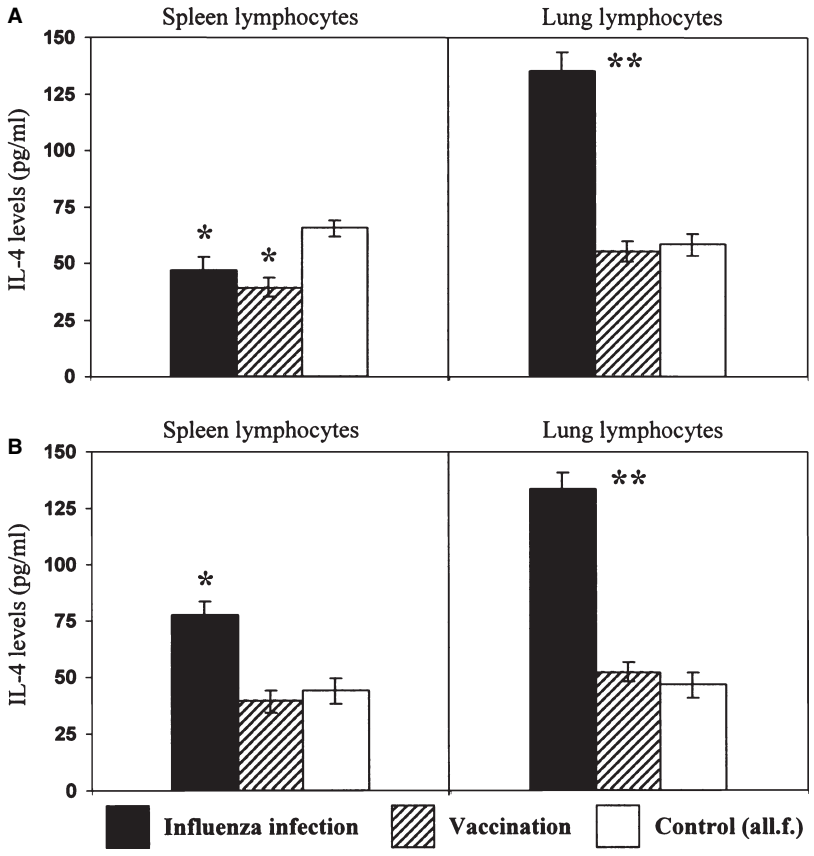

Figure 4. IL-4 production in mice surviving influenza infection or vaccination at acute asthma or at asthma remission phases. At the acute asthma (A) or at the asthma remission phase (B) mice were immunized with A/PR/8/34 influenza virus (infection) or Len/47 × PR/8 attenuated reassortant strain (vaccination) or allantoic fluid (control), and 14 days later were additionally exposed to i.n. OVA. At 48 hours after additional exposure to i.n. OVA spleen and lung lymphocytes were harvested and stimulated *in vitro* with 100 μg/ml OVA. *$P < 0{\cdot}5$, **$P < 0{\cdot}1$ compared with the control group.

attenuated reassortant showed no significant differences from the control group.

Pathologic changes in lungs

Histological examination of lungs was performed after virus administration and after subsequent exposure to OVA.

Acute asthma

In control mice (Figure 5A), the characteristic signs of acute asthma – obstructive emphysema and inflammatory eosinophilic infiltration were observed. Influenza infection during acute bronchial asthma led to a significant aggravation of inflammatory changes in the lungs (Figure 5B); edema of bronchial walls and dense peribronchiolar and perivascular infiltrates, consisting of eosinophils and neutrophils, were observed. Subsequent allergen (OVA) exposure provoked the development of even more pronounced inflammation (Figure 5E) with massive mononuclear infiltration. Practically, all the lung lobes were an entire inflammation focus; bronchial and alveolar lumens were filled with exudates consisting of cast-off epithelium cells, alveolar macrophages and erythrocytes.

Immunization with attenuated influenza virus during acute asthma also promoted inflammatory changes in the lung tissues (Figure 5C); large areas of peribronchiolar and perivascular infiltrates, consisting of eosinophils and neutrophils were present. However, after additional OVA exposure (Figure 5F), histological findings were not significantly different from that of the control (Figure 5D) and showed evidence of a progressive decrease in allergic inflammation where some fragments of emphysema and eosinophilic infiltration were replaced with normally structured lung parenchyma.

Asthma remission phase

The data of control mice (Figure 6A) indicated the decrease of allergic inflammation in lungs with marked reduction of infiltration and emphysema sites, this finding corresponded to the remission phase of asthma. Influenza infection at the asthma remission phase resulted in significant exacerbation of inflammatory and obstructive reactions in the lungs (Figure 6B). The appearance of dense mononuclear infiltrates, large areas of emphysema and local bleeding in alveoli has been noted. Pronounced inflammatory infiltration was observed even after additional exposure of infected animals to OVA (Figure 6E). Lung tissue inflammatory processes progressed with formation of granulomatous inflammation sites.

In contrast to infected mice, the lung histology of mice vaccinated at asthma remission provided evidence of minimal inflammatory changes after both virus administration (Figure 6C) and subsequent exposure to allergen

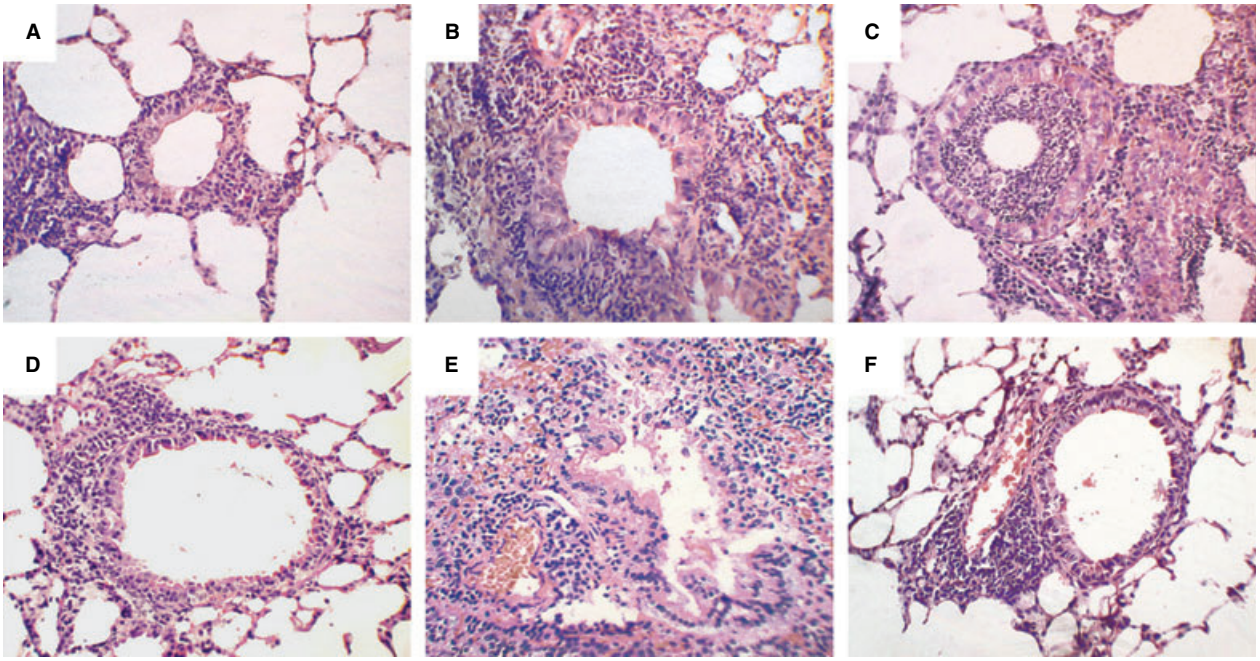

Figure 5. Lung histology of mice surviving influenza infection or vaccinated at the acute phase of asthma. Mice were treated as shown in Figure lA, lungs were stained with hematoxylin and eosin (×200). Mice were inoculated with: (A) all.f. instead of viruses (control group); (B) A/PR/8/34 influenza virus (infection); (C) Len/47 × PR/8 attenuated reassortant strain (vaccination). Then 14 day later mice were additionally exposed to OVA: (D) mice received a.f.; (E) mice survived influenza infection; (F) vaccinated mice.

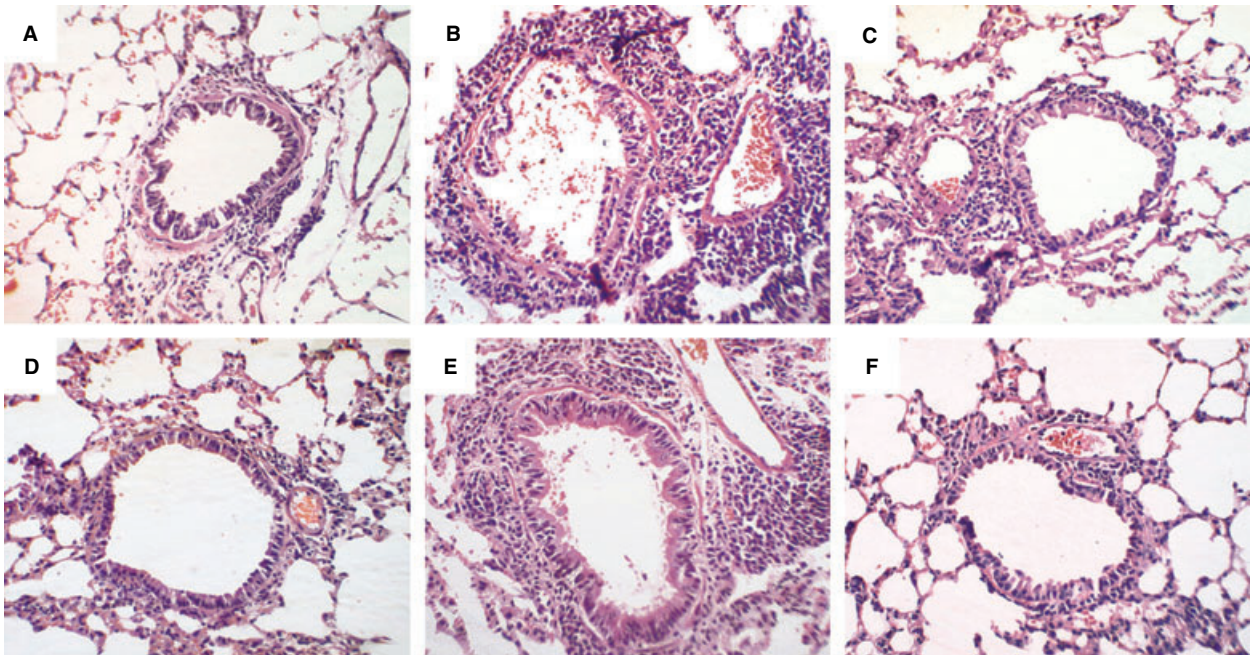

Figure 6. Lung histology of mice surviving influenza infection or vaccinated at the remission phase of asthma. Mice were treated as shown in Figure lB, lungs were stained with hematoxylin and eosin (×200). Mice were inoculated with: (A) allantoic fluid. instead of viruses (control group); (B) A/PR/8/34 influenza virus (infection); (C) Len/47 × PR/8 attenuated reassortant strain (vaccination). Then 14 days later mice were additionally exposed to OVA: (D) mice received allantoic fluid; (E) mice surviving influenza infection; (F) vaccinated mice.

(Figure 6F), and was little different from controls (Figure 6A, 6D) exhibiting normal structure of lung parenchyma.

Discussion

The role of influenza virus in development and exacerbation of respiratory tract allergic pathology has not been clarified fully. According to the "hygienic hypothesis," viral infections occurring in early childhood and inducing predominantly Thl-responses protect against allergic diseases.[23] On the other hand, many epidemiological studies suggest that influenza infection provokes serious exacerbations of bronchial asthma.[3–7] Investigation *in vivo* of the immunologic mechanisms underlying these controversial epidemiological phenomena requires specific animal models incorporating both bronchial asthma and influenza infection. Several *in vivo* experimental models are being used,[24] and the model of OVA-induced bronchial asthma in mice is the most widespread. Studies based on this model have shown that influenza infection is able to aggravate allergen-specific Th2-immune response in OVA-sensitized mice significantly.[25–28] However, in these studies the OVA-dependant asthma was performed after infection. In nature, asthmatic subjects contact the influenza virus when their bronchial asthma has already formed and when asthma is of various levels of severity, which is the situation we tried to reflect in the present study.

Application of OVA as an allergen allows the induction of acute bronchial asthma which, nevertheless, is a transitory one.[24] In support we have confirmed a marked decrease of respiratory tract allergic inflammation to 10 days after the final OVA inoculation. Therefore, due to the chosen model's peculiarities, we were able to single out two stages of OVA-induced bronchial asthma with various inflammation severities, an acute asthma phase and a remission phase. At these time points we have compared the effects of influenza infection and immunization with an attenuated reassortant virus as well as their influence on subsequent additional exposure of animals to OVA.

We have demonstrated that influenza infection provokes the exacerbation of OVA-induced bronchial asthma at any stage of its development and significantly increases inflammatory cell infiltration to the lungs. Asthma exacerbation was also observed after subsequent OVA administration to the same mice, but an unexpected decrease in OVA-specific IgE has been noted after an additional allergen stimulation of the animals that survived infection during the acute asthma phase. A transient decrease in OVA-specific IgE production after the influenza infection in OVA-sensitized animals was observed in earlier studies by other authors.[26] Possibly, this is related to the suppression of IL-4 production in spleen lymphocytes, as IL-4 is a main cytokine switching B-cells to IgE synthesis.[29] IL-4 levels in lung lymphocyte cultures were very high, which correlated with the intensity of

local allergic inflammation that was found in histological slides. Overall, our results agree with recent studies demonstrating the aggravation of allergen-specific Th2-mediated immune response to influenza infection.[26–28,30]

Immunization of mice with attenuated reassortant virus at the acute asthma phase also led to an enhancement of asthma signs in the lung. However, in contrast to infection, immunization with attenuated reassortant during the bronchial asthma remission phase did not result in an increase of OVA-specific serum IgE levels, or produce marked lung inflammation. Additional allergen administration to vaccinated asthmatic animals did not interfere with immunological or histological changes.

The difference in pathogenic and attenuated virus effects on OVA-sensitized mice is obviously specified by peculiarities of virus reproduction in respiratory tract. The attenuated reassortant possesses *ca*- and *ts*-phenotypes and can multiply only in the upper respiratory tract but not in the lungs. In contrast to the attenuated strain, pathogenic virus affects all of the respiratory tract including the lungs. Recently, it has been shown that exacerbation of allergen-specific Th2-immune response after influenza virus administration is mediated by pulmonary dendritic cells (DC). Yamamoto *et al.*[27] have demonstrated that influenza infection through the recruitment of DC in the lungs activates allergen capture and presentation and consequently provokes asthmatic symptoms in mice which survived infection and then were exposed to OVA. Moreover, enhancement of allergen-specific Th2-immune response by pulmonary DC can have an IFNγ-dependent mechanism and directly coincides with the intensity of Thl-mediated response to influenza virus.[30] Influenza virus reproduction in the lungs also promotes retention of DC in airways[27] and increases the recruitment of allergen-specific memory Th2-cells.[28] These data indicate that influenza virus is capable of enhancing allergen-specific immune response and consequently asthma exacerbation because of multiplication in the lower respiratory tract. In agreement with this hypothesis, our results show that additional OVA administration provokes asthma exacerbation only in mice which survived influenza infection but not in vaccinated animals, even when mice were exposed to OVA again after complete virus clearance. These findings also coincide with the recent study of Tsitoura *et al.*[16] who demonstrated that influenza infection disrupts the induction of allergen-specific T-cell tolerance. Attenuated virus having a different reproduction site in the airways appears to be incapable of altering OVA-unresponsiveness and to be safe in relation to additional OVA administration to asthmatic mice.

In summary, our results clearly indicate that influenza infection provokes asthma exacerbation regardless of disease phase (acute asthma or remission) and even after complete virus clearance induces the aggravation of airway inflammation under additional allergen exposure. Vaccination with live attenuated reassortant influenza virus leads to asthma exacerbation only when virus immunization occurs during the acute asthma phase. In contrast to infection, vaccination during the remission phase of bronchial asthma is safe and does not enhance allergic inflammatory changes. Vaccination with live attenuated virus also does not interfere upon subsequent contact of asthma sufferers with allergen.

Conflict of Interest

BioDiem Ltd., Melbourne, Australia, provided financial support for this study.

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