

Respiratory syncytial virus, pneumonia virus of mice, and influenza A virus differently affect respiratory allergy in mice

M. Barends*[‡], L. G. H. de Rond*, J. Dormans[†], M. van Oosten*, A. Boelen*, H. J. Neijens[‡], A. D. M. E. Osterhaus[§] and T. G. Kimman*

*Laboratory of Vaccine-Preventable Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands, [†]Laboratory for Toxicology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands, [‡]Department of Paediatrics, Erasmus MC, Rotterdam, The Netherlands and [§]Institute of Virology, Erasmus MC, Rotterdam, The Netherlands

Summary

Background Respiratory viral infections in early childhood may interact with the immune system and modify allergen sensitization and/or allergic manifestations. In mice, respiratory syncytial virus (RSV) infection during allergic provocation aggravates the allergic T helper (Th) 2 immune response, characterized by the production of IL-4, IL-5, and IL-13, and inflammatory infiltrates. However, it is unclear whether the RSV-enhanced respiratory allergic response is a result of non-specific virus-induced damage of the lung, or virus-specific immune responses.

Objective In the present study we investigated whether RSV, pneumonia virus of mice (PVM) and influenza A virus similarly affect the allergic response.

Methods BALB/c mice were sensitized and challenged with ovalbumin (OVA), and inoculated with virus during the challenge period. Pulmonary inflammation, lung cytokine mRNA responses, and IgE production in serum were assessed after the last OVA-challenge.

Results Like RSV, PVM enhanced the OVA-induced pulmonary IL-4, IL-5, and IL-13 mRNA expression, which was associated with enhanced perivascular inflammation. In addition, PVM increased the influx of eosinophils in lung tissue. In contrast, influenza virus decreased the Th2 cytokine mRNA expression in the lungs. However, like PVM, influenza virus enhanced the pulmonary eosinophilic infiltration in OVA-allergic mice.

Conclusion The Paramyxoviruses RSV and PVM both are able to enhance the allergic Th2 cytokine response and perivascular inflammation in BALB/c mice, while the Orthomyxovirus influenza A is not.

Keywords allergy, eosinophils, influenza A, mice, PVM, RSV, Th2 cytokines

Submitted 24 April 2003; revised 25 July 2003; accepted 17 November 2003

Introduction

Childhood asthma is a serious global public health problem. According to the World Health Organisation [1], asthma is the most common chronic disease in children. Allergic asthma is a T helper 2 (Th2)-associated airway inflammation, characterized by the infiltration of eosinophils, and production of IL-4, IL-5, and IL-13 by Th2 lymphocytes in response to certain antigens [2, 3]. Although no single specific cause of asthma has been found, a number of pre-disposing factors has been identified that are targets of ongoing clinical investigation. One potential risk factor is viral respiratory tract infection in infancy, especially respiratory syncytial virus (RSV) [4, 5].

RSV (a member of the pneumovirinae subfamily of the *Paramyxoviridae*) is the most frequent cause of bronchiolitis

and pneumonia in infants and young children. The symptoms vary from mild upper respiratory tract illness to severe bronchiolitis and pneumonia [6]. In mice, RSV infection enhances the pulmonary Th2 cytokine expression and eosinophilic infiltration induced by airway sensitization and challenge with ovalbumin (OVA) [7, 8]. Recently, we showed that the RSV-induced Th1 response, characterized by IFN- γ and IL-12 production, occurred simultaneously with enhancement of the OVA-induced Th2 response [7]. However, in the absence of virus-induced IFN- γ , the Th2 enhancement was much stronger [9]. It is unclear whether the RSV-enhanced respiratory allergic responses in mice are a result of non-specific or virus-specific stimuli. For this reason, we investigated the influence of other respiratory viruses on the allergic immune response.

The natural rodent pathogen, pneumonia virus of mice (PVM), is also a member of the *Pneumovirinae* subfamily of the *Paramyxoviridae*, and is related to human RSV and the recently identified human metapneumovirus (hMPV) [10]. Molecular cloning has shown that the organization of the PVM genome is similar to that of (human) RSV [11].

Correspondence: Dr. T.G. Kimman, Laboratory for Vaccine-Preventable Diseases, National Institute of Public Health and Environment, PO Box 1, 3710 BA Bilthoven, The Netherlands. E-mail: tg.kimman@rivm.nl

Restricted serological cross-reactivity between the nucleocapsid protein (N) and the phosphoprotein (P) of PVM and RSV has been described, but none of the external proteins show cross-reactivity, and the viruses can be distinguished from each other in neutralization assays [12].

Like RSV, influenza virus (a member of the *Orthomyxoviridae* virus family) can induce inflammation in humans throughout the respiratory tract [13]. Mouse-adapted influenza virus can induce a lower respiratory tract infection, and the pathology in mice is comparable with that in humans [13]. Experimental studies in mice demonstrated that influenza virus infection enhanced the sensitization for allergic responses later in life [14, 15]. Infection with influenza A virus is also associated with asthma exacerbation in humans [16, 17]. Here we study the effect of influenza virus infection on an already existing respiratory allergy in mice. We thus investigated whether the RSV-enhanced respiratory allergy is restricted to RSV, or is also induced by other paramyxoviruses and non-paramyxoviruses, such as the *Orthomyxoviridae*. We therefore sensitized and challenged mice with ovalbumin (OVA) and infected them during the OVA challenge period with RSV, PVM, or influenza A virus. Subsequently, we determined the pulmonary inflammation, serum IgE, and lung cytokine mRNA responses. The results indicate that in our allergy model, the paramyxoviruses RSV and PVM enhance allergic Th2 cytokine responses while the orthomyxovirus influenza A does not.

Methods

Viruses

Human respiratory syncytial virus type A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The virus was cultured on HEp-2 cells (ATCC, Rockville, MD, USA) in medium (RPMI 1640 Gibco BRL, Life Technologies, Rockville, MD, USA), containing 10% heat-inactivated foetal calf serum (FCS, Greiner, Frickenhausen, Germany), 2 mM glutamine, 100 IE/mL penicillin, and 100 U/mL streptomycin. After 40–44 h of culture, the cells were sonicated and centrifuged for 10 min at 1000 g, 4 °C. The supernatant was collected and 50% PEG₆₀₀₀ (Merck, Darmstadt, Germany) in 150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.5, was added to a final concentration of 10%. After incubation for 1.5 h at 4 °C (while stirring), the suspension was centrifuged for 15 min at 3200 g, 4 °C. The pellet was resuspended in culture medium in 10% of the original volume. The suspension was aliquotted, snap-frozen, and stored at –80 °C. The virus

stock contained 3×10^8 plaque forming units (pfu) RSV/mL as assessed by quantitative plaque-forming assay [18].

Pneumonia virus of mice (PVM, a gift of Dr. Elzinga-Gholizadea, Bilthoven, the Netherlands) was grown and quantified by virus titration on BHK-21 C₁₃ cells in medium (Eagle MEM, containing Hanks salts, 10 mM NaHCO₃, 10% FCS, 2 mM glutamine, 20 mM Hepes, 100 IE/mL penicillin, and 100 U/mL streptomycin).

Influenza A virus (mouse adapted strain A/PR/8/34 (H1N1) was grown and quantified by virus titration on MDCK-1 cells in medium (Eagle MEM, containing Hanks salts, 10 mM NaHCO₃, 2 mM glutamine, 13 mM Hepes, 100 IE/mL penicillin, 100 U/mL streptomycin, $10^{-5}\%$ trypsin (Merck), and 10% PGR-Albumin (containing 10 g Pepton, 10 g Glucose and 10 g Bovine albumine per 800 mL Eagle MEM). The influenza A and PVM virus stocks were prepared as described for RSV (see above).

Animals

Female SPF BALB/c mice were obtained from Harlan Olac (Horst, the Netherlands) and were used at 6–10 weeks of age. A week before the experiments started, mice were housed per group according to the experimental set-up, and cages were placed in a temperature-controlled isolator (22 °C) to prevent infection of control animals and animal caretakers. Mice were kept in a 12-h light/dark cycle, and received food and water *ad libitum*. The study was approved by the Institute's committee on animal welfare.

Experimental design

Mice were sensitized to OVA by intraperitoneal (i.p.) administration of 0.5 mL OVA (20 µg/mL saline, no adjuvant (Sigma-Aldrich, Steinheim, Germany)) for 7 times on every other day starting at day 0. From day 33 till day 40, mice were intranasally challenged with OVA aerosols (2 mg/mL saline) for 5 min on 8 consecutive days (Fig. 1).

To examine the influence of the different viral infections on a pre-existing allergic inflammation, OVA-sensitized mice were inoculated intranasally with 50 µL RSV (1.5×10^7 plaque forming units per dose $\sim 8.10^6$ TCID₅₀), 50 µL PVM (20 TCID₅₀ per dose), or 50 µL influenza virus (50 TCID₅₀ per dose), on day 35 during the OVA-challenge period (= OVA/RSV, OVA/PVM, or OVA/influenza). A pilot study was performed in which day 35 was determined to be the optimal day for inoculation during OVA-challenge (data not shown). The lowest infection dose that still caused clinical symptoms was chosen (data not shown). Virus-infected OVA-allergic mice were compared with OVA-allergic mice inoculated with their respective uninfected culture lysate

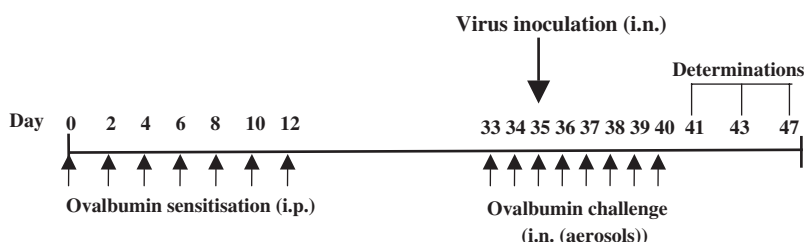


Fig. 1. Study design.

(mock) (= OVA/Mock-r, OVA/Mock-p, OVA/Mock-i), and non-allergic mice infected with virus only (= -/RSV, -/PVM, -/Influenza). Before inoculation, mice were i.p. anaesthetized with Ketamine, Rompun (= xylazine), and Atropine (KRA). At days 41, 43, and 47 of the experimental protocol (days 6, 8, and 12 days post virus inoculation), mice were anaesthetized with KRA i.p. and sacrificed. Three different time points for sampling were chosen in order to detect a prolonged inflammatory response. Blood was collected via orbital puncture. The lungs were removed. The right lung was snap frozen in liquid nitrogen and stored till further process. The left lung was fixed intratracheally with formalin, and subsequently immersed in fixative. Each experiment was performed in duplicate, and gave similar results. Data of one experiment are shown.

Histopathologic examination

Formalin-fixed lungs were embedded in paraplast (Monoject, Kildare, Ireland). Transverse sections of 5 µm were stained with haematoxylin and eosin. Different lung lesions were scored semiquantitatively from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

Immunohistochemistry

Immunostaining of PMV, RSV and influenza virus was done on paraffin sections of lung. Deparaffinized sections were incubated with respectively rabbit anti-PVM antibody (gift from G. Drost, RIVM), goat anti-RSV (Biodesign International, Saco, Maine, US), and mouse anti-HB-65 antibody, (mIgG2a, directed against nucleoprotein of influenza virus, gift from Dr. A. Meyer, RIVM). The following peroxidase labelled secondary antibodies were used: swine anti-rabbit IgG (DAKO Diagnostics, Glostrup, Danmark), donkey anti-goat IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA), and goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, Alabama, USA). Primary and secondary antibodies were diluted in PBS containing 1% Bovine Serum Albumine (Sigma, Zwijndrecht, the Netherlands) and used in an optimal dilution. Sections were washed in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany). Detection of peroxidase occurred with the substrate 3,3-diaminobenzidine (Sigma, Zwijndrecht, the Netherlands) with ammonium nickel sulphate (Fluka Chemie GmbH, Buchs, Switzerland). Cells were counterstained with nuclear fast red (Sigma, Zwijndrecht, the Netherlands).

RNA isolation and RT-PCR

Total cellular RNA was isolated from frozen lung samples using Trizol Reagent (GibcoBRL, Life technologies, Rockville, USA). Five micro liter of total lung RNA was reverse transcribed, cytokine PCR reactions (IL-4, IL-5, IL-13, IFN-γ, and IL12) on the cDNA, and analysis of PCR products were carried out as described previously [9, 18]. Eotaxin RT-PCR was performed according to the same protocol using sense (CCCCAACACACTACTGAAGA) and anti-sense (CTA CATGAAGCCAAGTCCTT) primers. Macrophage inflammatory protein-1α (MIP-1α) RT-PCR was carried out as described by Su et al. [19].

IgE antibodies in serum

Total and OVA-specific IgE antibodies in serum were determined using a modified capture ELISA as described previously [9].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance for differences in cytokine ratios and IgE concentrations was determined using the Student's *t*-test (Excel, Microsoft Corporation, Redmond, USA), and for histological scores using the non-parametric Wilcoxon's test (SAS, SAS Institute Inc., USA).

Results

Kinetics of virus replication

Differences in replication kinetics between RSV, PVM, and influenza virus might have consequences for their effect on respiratory allergy in mice. We therefore determined the virus titres of RSV, PVM and influenza A virus in lung tissue of non-allergic mice on days 4, 5 and 6 post inoculation (Fig. 2). RSV reached a peak titre on day 4 post inoculation, influenza virus on day 5 post inoculation, and the PVM titre was still increasing at day 6 post inoculation. The peak titres of influenza and PVM were approximately 1–2 log higher compared with that of RSV.

Pulmonary histopathology

In mice, OVA-sensitization and OVA-challenge induced significant peribronchiolar (data not shown) and perivascular (Figs 3a–c) infiltration of eosinophilic and mononuclear cells. In addition, OVA-allergy induced a marked hypertrophy of the mucus-producing epithelial cells.

RSV infection in non-sensitized/challenged mice resulted in a slight peribronchiolar infiltration of mononuclear cells and hypertrophy of the mucus-producing cells, together with marked perivascularitis and alveolitis (Fig. 3a). PVM and influenza virus induced similar lesions in lungs of

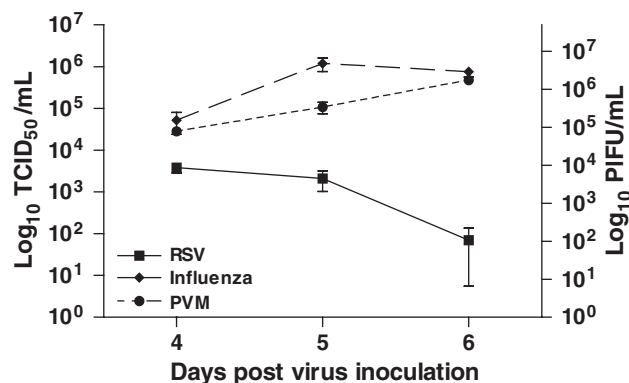


Fig. 2. Respiratory syncytial virus (RSV), Pneumonia virus of mice (PVM), and influenza A virus titres in lung homogenates of BALB/c mice on day 4, 5 and 6 post inoculation. The virus titres are expressed as log₁₀ TCID₅₀/lung for PVM and influenza A (left y-axis), and log₁₀ pfu/lung for RSV (right y-axis). Mean values ± SEM (*n* = 6) are shown.

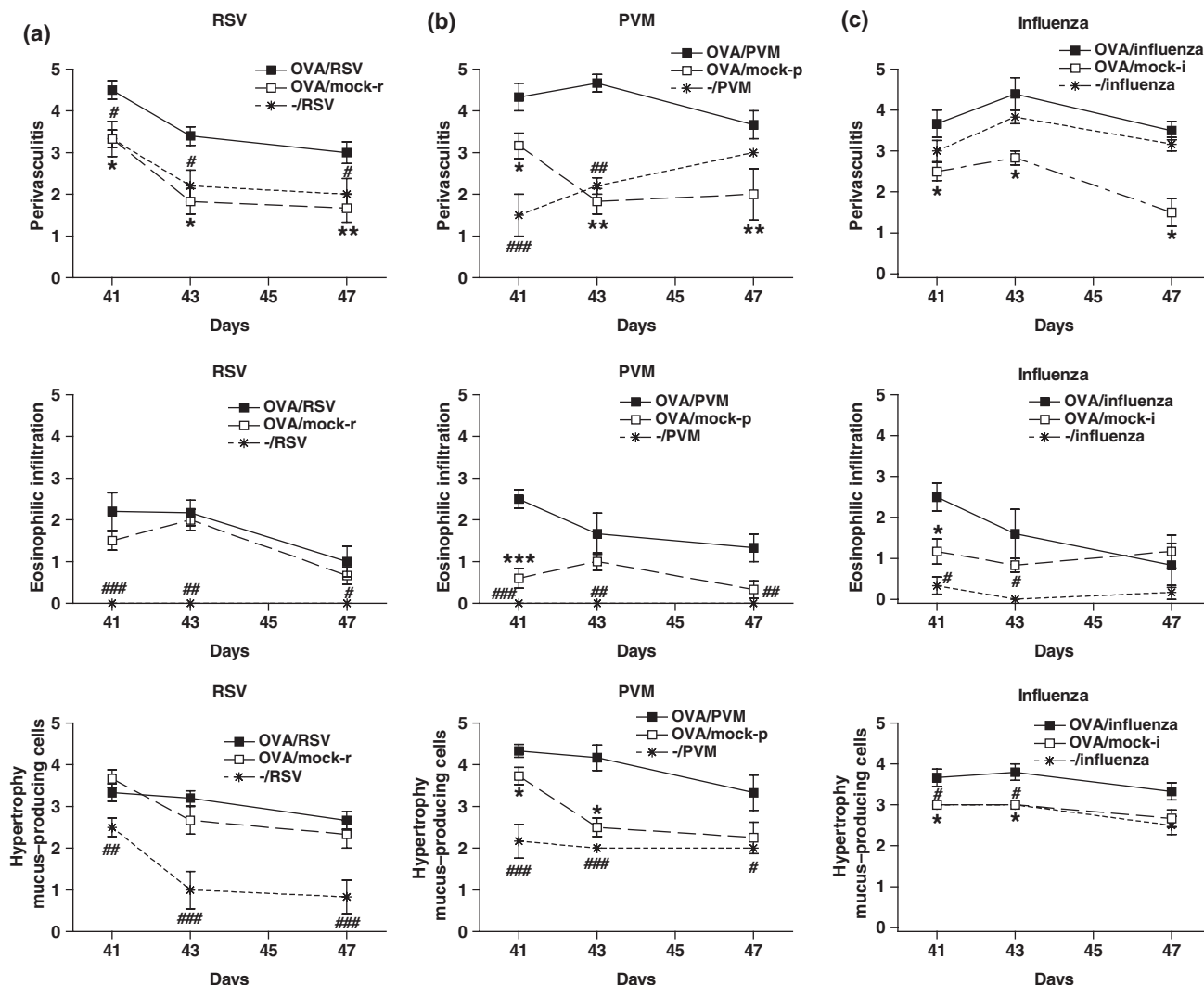


Fig. 3. Histopathologic changes in lungs examined on day 41, 43 and 47 (is 1, 3 and 7 days after the last ovalbumin (OVA)-challenge). (a) OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). (b) OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). (c) OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Pulmonary lesions are scored from minimal (1) to severe (5). Histological scores are depicted as mean value $\pm$ SEM ($n = 6$). Statistical difference on one single time-point between differently treated groups is analyzed using the non-parametric Wilcoxon's test; differences are indicated by * $P < 0.05$, ** $P < 0.01$ between OVA/virus and OVA/mock, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ between OVA/virus and -/virus.

non-sensitized/challenged mice as RSV (Figs 3b and c). However, the pathological changes in PVM and influenza virus infected mice were still marked on day 12 post inoculation (is day 47 of the experimental protocol), while those in RSV infected mice were already reduced to slight lesions. None of the virus-infected non-allergic mice showed eosinophilic inflammation in the lungs (Fig. 3).

The histopathologic changes in lungs of OVA-allergic mice infected with RSV, PVM or influenza virus were compared with changes in the corresponding OVA/mock group (Fig. 3). PVM and RSV infection increased the perivascularitis in OVA-allergic mice. Influenza virus did not further enhance perivascularitis in OVA-allergic mice. In fact, perivascularitis was similar in allergic and non-allergic mice, infected with influenza virus. Influenza virus and PVM enhanced the hypertrophy of the mucus producing cells in OVA-allergic mice on day 41 and 43, while RSV did not enhance it at any

time point (Fig. 3). PVM and influenza virus significantly increased the influx of eosinophils in allergic mice on day 41 (1 day after the last OVA-challenge) (Fig. 3). Remarkably, RSV did not significantly enhance the influx of eosinophils in OVA-sensitized/challenged mice as we have seen before [7].

Localization of RSV, PVM, and influenza virus antigen in lung tissue

RSV was detected mainly in the cytoplasm of alveolar epithelium and to a lesser extent in alveolar macrophages at day 6 post inoculation (day 41 of the experimental protocol) (Fig. 4a). In the epithelium, both alveolar epithelial cells of type I (characterized by thin long cytoplasmic extensions) and type II (cubic cells) stained positive for RSV. OVA-allergy induced no difference in cell types staining positive for RSV at day 6 post inoculation (Figs. 4a and b). At day 8, only in

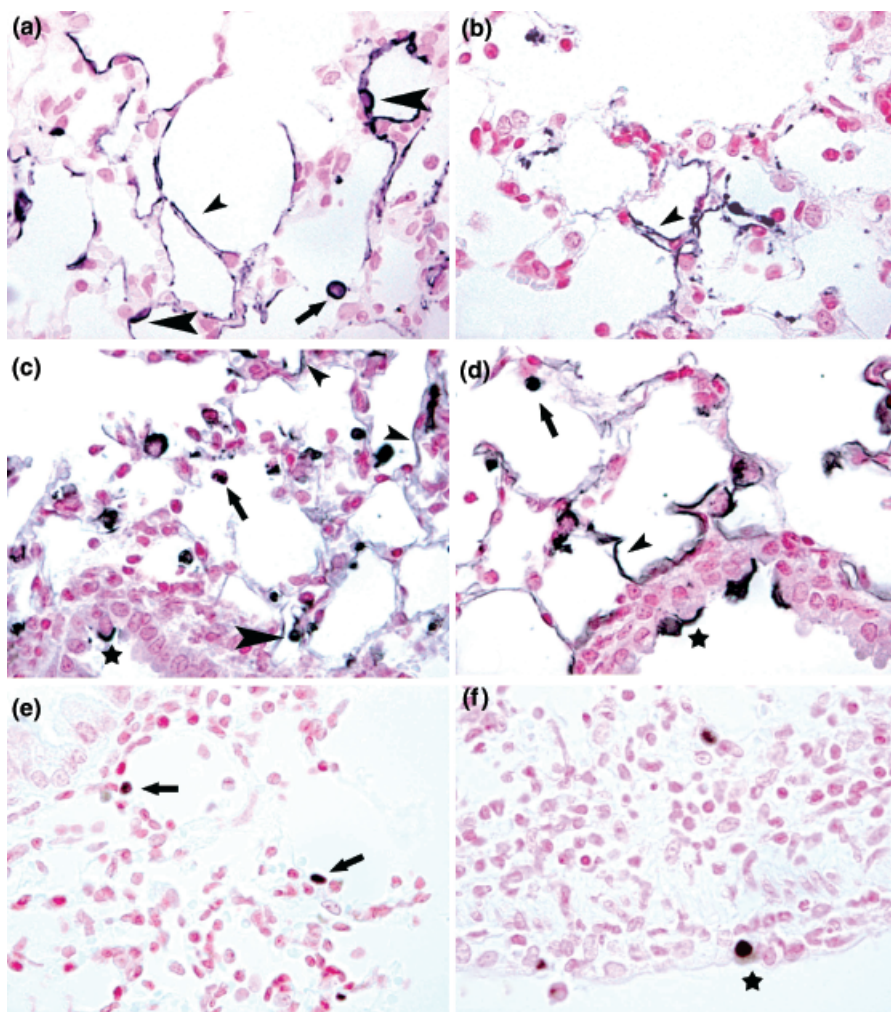


Fig. 4. Sections of lungs from mice intranasally inoculated with RSV (a, b), PVM (c, d) and influenza (e, f) with (a, c, e) or without (b, d, f) ovalbumin treatment are shown. All pictures represent immunoperoxidase staining 6 days post virus inoculation (1 day after the last OVA-challenge). Sections were counterstained with nuclear fast red, and examined using light microscopy (660 $\times$). Immunostaining for RSV is observed in alveolar macrophages (arrow in a), alveolar type I (small arrowhead in a, b) and type II epithelial cells (large arrowhead in a), independently of the OVA-treatment. Immunoreactivity for PVM is seen in the cytoplasm of bronchiolar cells (non-ciliated, non-mucus-secreting epithelial cells, asterisk in c, d), in alveolar type I (small arrowhead in c, d), and type II epithelial cells (large arrowhead in c), and alveolar macrophages (arrow in c, d), independent of OVA-treatment. Alveolar macrophages (arrow in e) and bronchiolar cells (asterisk in f) stained positive for influenza independent of ovalbumin treatment.

OVA-allergic mice, a few alveolar macrophages were still RSV-positive and no epithelial cells stained positive, while at day 12 no RSV-positive cells were present at all, both in OVA-allergic and non-allergic mice (data not shown).

PVM was detected mainly in the cytoplasm of bronchiolar and alveolar epithelial cells of both type I and type II, as well as in alveolar macrophages at day 6 post inoculation (Fig. 4c). OVA-allergy induced no differences in cellular localization of PVM (Figs. 4c and d). At day 8 post PVM-inoculation, the number of PVM-positive cells in the OVA-allergic mice was lower than on day 6, while in the non-allergic mice PVM-positive cells were nearly absent (data not shown). At day 12 post inoculation, no PVM-positive cells were observed in both OVA-allergic as non-allergic mice (data not shown).

Influenza virus was only detected in some solitary bronchiolar cells (non-ciliated, non-mucus-secreting epithelial cells) and alveolar macrophages, but not in alveolar epithelial cell, as examined on day 6 post inoculation (Fig. 4e). No difference in cell types staining positive for influenza was noticed between OVA-allergic and non-allergic mice (Figs 4e and f). At days 8 and 12 post inoculation no influenza-positive cells could be demonstrated in both OVA-allergic as non-allergic mice (data not shown).

Th2 cytokines in lung tissue

OVA sensitization and challenge induced a predominant Th2 cytokine milieu in lung tissue, characterized by significant increases in IL-4, IL-5, and IL-13 mRNA expression on day 41 (1 day after the last OVA-challenge) when compared with non-allergic mice (Fig. 5). As shown before [7], RSV in non-allergic mice induced no or minimal mRNA expression of the Th2 cytokines IL-4, IL-5, and IL-13 (Fig. 5a). Also PVM and influenza virus infection in non-allergic mice did not induce mRNA expression of these Th2 cytokines (Figs 5b and c).

RSV infection during OVA-challenge significantly increased the OVA-induced pulmonary IL-4 mRNA expression till at least day 47, and the IL-5, and IL-13 mRNA expression on day 43 of the experimental protocol (Fig. 5). Like RSV, PVM infection during OVA-challenge strongly enhanced the pulmonary IL-4, IL-5, and IL-13 mRNA expression when compared with OVA/Mock-p mice (Fig. 5). In contrast, influenza A virus infection did not enhance mRNA expression of IL-4, IL-5 and IL-13 in OVA-allergic mice (Fig. 5c). Influenza virus infection even down-regulated mRNA expression on day 41 of all three Th2 cytokines tested compared with OVA/mock-i mice.

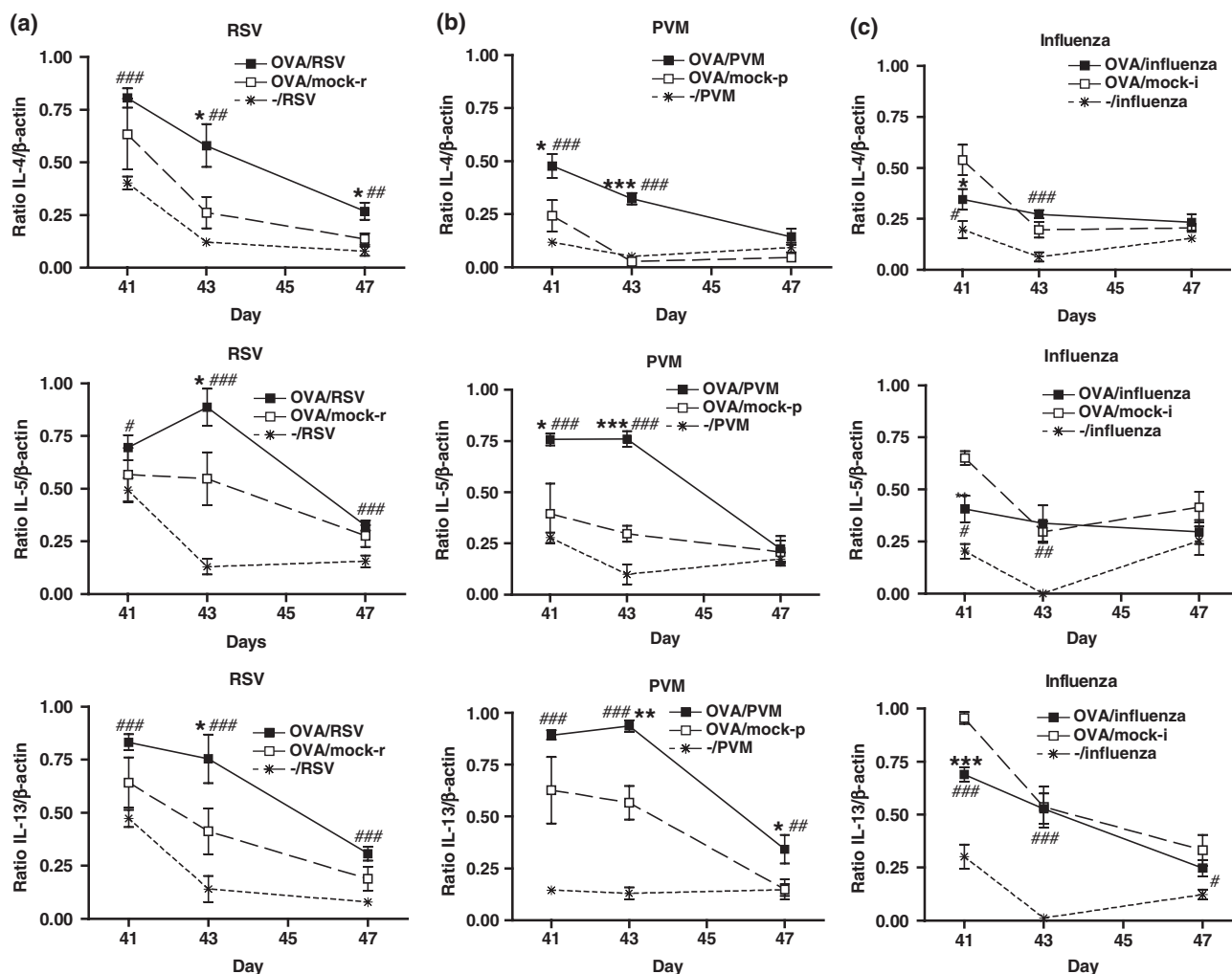


Fig. 5. Relative expression (against β -actin) of lung IL-4, IL-5 and IL-13 mRNA determined at days 41, 43 and 47 of the experimental protocol (is 1, 3 and 7 days after the last OVA-challenge). (a) OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). (b) OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). (c) OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Mean values $\pm$ SEM ($n = 6$ per group) are depicted. Statistical differences are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between OVA/virus and OVA/mock, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ between OVA/virus and -/virus.

Chemokine expression in lung tissue

The decrease in pulmonary IL-4, IL-5, and IL-13 mRNA expression in OVA/influenza mice on day 41 compared with OVA/mock-i mice, seems in contrast with the influenza virus enhanced eosinophilic inflammation, since IL-5 is considered important for eosinophilic infiltration. Besides IL-5, the β -chemokines eotaxin and MIP-1 α are also involved in eosinophilic inflammation [3]. MIP-1 α can induce infiltration of eosinophils in lungs independent of IL-5 [20]. OVA-sensitization/challenge without virus infection resulted in increased expression of pulmonary eotaxin mRNA, but had no effect on MIP-1 α mRNA expression, compared with non-infected non-allergic controls. RSV, PVM and influenza virus infection of non-allergic mice also induced strong expression of eotaxin and MIP-1 α mRNA (Fig. 6). When OVA-allergic mice were infected with one of the viruses, only PVM enhanced the expression of eotaxin mRNA, while influenza virus even down-regulated eotaxin mRNA expression on day

41. The high expression of pulmonary MIP-1 α mRNA in RSV-, PVM- or influenza virus-infected mice was not altered by OVA-sensitization/challenge (Fig. 6).

Th1 cytokine mRNA expression

IFN- γ and IL-12 are the major Th1 cytokines. Both are associated with antiviral activity and reduce Th2-like responses. OVA-treatment without viral infection did not result in the expression of Th1 cytokines. RSV, PVM and influenza virus infection induced high expression levels of IFN- γ and IL-12 mRNA in lung tissue up to at least 12 days post inoculation (day 47 of the experimental protocol) (data not shown). The expression of IFN- γ and IL-12 induced by RSV, PVM or influenza virus was not reduced by OVA-sensitization/challenge (data not shown). PVM infection in OVA-sensitized/challenged mice even enhanced expression of

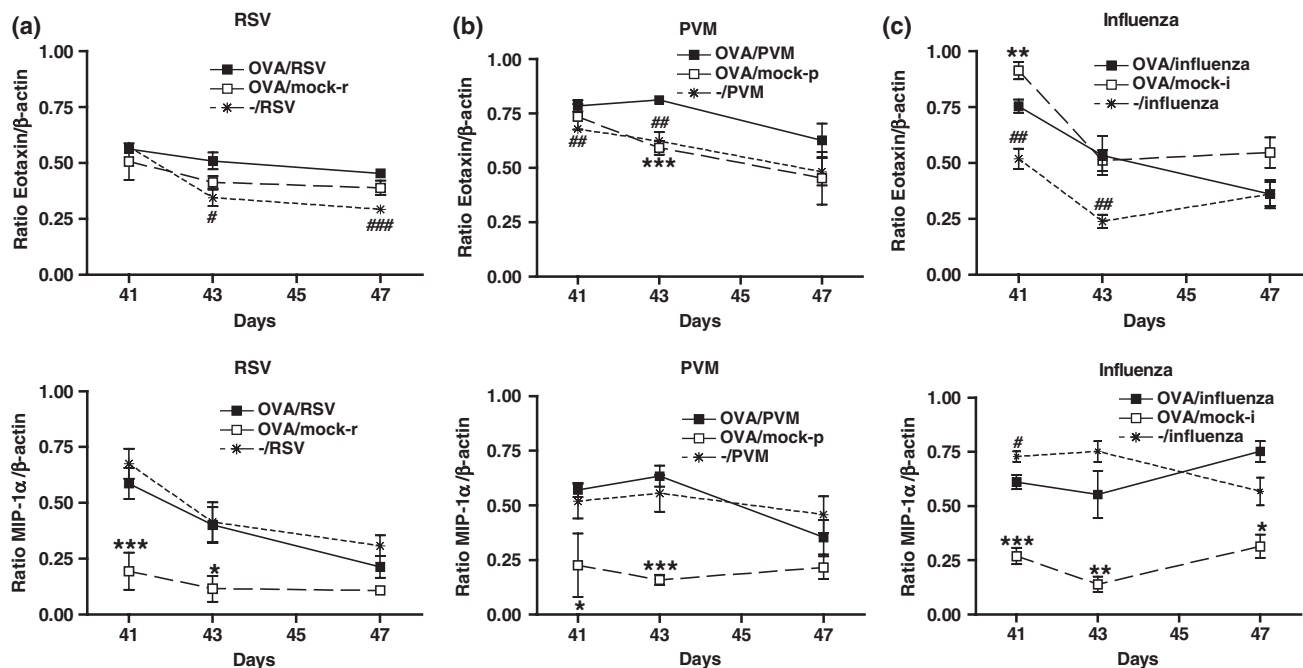


Fig. 6. Relative expression (against β -actin) of lung eotaxin and MIP-1 α mRNA determined at day 41, 43 and 47 of the experimental protocol (is 1, 3 and 7 days after the last OVA-challenge). (a) OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). (b) OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). (c) OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Mean values $\pm$ SEM ($n = 6$ per group) are depicted. Statistical differences are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between OVA/virus and OVA/mock, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ between OVA/virus and -/virus.

IL-12 on day 43 compared with non-sensitized/challenged mice infected with PVM.

IgE in serum

We previously reported that OVA sensitization and challenge resulted in the production of OVA-specific IgE in serum, measured after the OVA-challenge period. In addition, RSV infection during the OVA-challenge period did not affect titres of OVA-specific IgE and total-IgE in serum [7]. In the present series of experiments none of the virus infections influenced the total- and OVA-specific IgE production in OVA-sensitized/challenged mice, at least till day 47 of the experimental protocol (data not shown). In serum of non-allergic mice infected with PVM, RSV, or influenza virus, no detectable levels of total-IgE could be measured.

Discussion

In mice, RSV infection aggravates the effects of allergic sensitization and challenge, predominantly by an increase in Th2 cytokine mRNA expression in the lungs [7] and hyper-reactivity of the airways [8]. However, it is not clear how RSV enhances the allergic response and whether RSV is unique in its ability to enhance the inflammatory and cytokine responses in allergic mice. We therefore studied the influence of infection with two other respiratory viruses, PVM and influenza A virus.

PVM is closely related to RSV and belongs to the same virus family; the *Pneumovirinae*. Because RSV is not a murine pathogen, large doses are required to infect mice and infection

results in little clinical disease [6, 21]. In contrast, mice are more sensitive to PVM than to RSV infection (this article, [22]). Therefore, PVM has been used as a model to study the pathology and immune responses of RSV in humans [23, 24]. The data presented in this article indicate that PVM infection of allergic mice is an even better model to study the effects of respiratory virus infection on allergy than RSV.

Like RSV, influenza A virus infection is associated with asthmatic exacerbations in humans [16, 17] and enhanced allergic sensitization in mice [14, 15].

RSV, PVM and influenza A virus infection of mice result in a dominant Th1 like cytokine response in lungs, together with alveolar, peribronchiolar and perivascular inflammation. OVA-sensitization/challenge did not affect the virus-induced Th1-cytokine expression. The virus-induced lung pathology and expression of pulmonary IFN- γ and IL-12 mRNA decreased more rapidly in RSV-infected, than in PVM and influenza-infected mice. These differences could be due to differences in virus-replication kinetics. Like RSV, PVM infection of allergic mice increased the expression of pulmonary IL-4, IL-5, and IL-13 mRNA and enhanced the perivascularitis and infiltration of eosinophils in the peribronchiolar space. In contrast, influenza virus infection did not enhance the pulmonary Th2 cytokine mRNA expression in OVA-allergic mice, but even down-regulated the IL-4, IL-5, and IL-13 mRNA response on day 41. These results appear in contrast to other published data, which demonstrate that influenza virus infection enhances the Th2 inflammatory response in allergen-sensitized mice [14, 15]. However, these studies examined the influence of infection on later development of allergic sensitization rather than the effects of infection on a pre-existing allergy, like in our study. We

cannot exclude that influenza virus infection on another moment during the OVA-sensitization and -challenge protocol in our mice model may influence the allergic Th2 immune response.

The key question is why infection with RSV and PVM enhance the OVA-induced Th2 cytokine response, while that with influenza virus does not. The difference in Th2-enhancement by the three viruses might be explained by the difference in virus-structure between the involved virus families. A characteristic outer membrane protein of orthomyxoviruses is the HA protein, which is involved in attachment of virus to host cells and hemagglutination [25]. For attachment to host cells PVM and RSV are equipped with the G-protein [6]. G is extensively glycosylated, and lacks homology to the HA protein or to any other known paramyxovirus protein [26]. A possible mechanism for influencing the OVA-induced Th2 cytokine response could be the immune-modulating capacity of the pneumovirus G protein. Immunization with purified G protein or vaccinia virus vectors expressing G, stimulate a Th2 immune response [27–29]. The OVA-induced pulmonary inflammation and Th2 cytokine milieu in mice might favour the immune response to G and further stimulate the Th2-inflammatory response [26]. In contrast, influenza virus seems to lack a Th2-stimulating protein. This hypothesis can be investigated by inoculation with G protein only and study the OVA-allergic Th2 immune response.

A second possible mechanism that may explain the difference between the pneumoviruses and influenza virus in enhancing the OVA-induced Th2 cytokine response could be the site of virus replication in lung tissue. As demonstrated, PVM and RSV were mainly located in the epithelial cells lining the alveoli and bronchi, while influenza virus was predominantly observed in the alveolar macrophages. In addition, PVM and RSV were detected during a longer period of time in lungs of allergic mice than non-allergic mice, while influenza virus was not. The absence of influenza virus replication in alveolar epithelial cells is in contrast to previously published data [30, 31]. However, in the present study mice are inoculated with the A/PR/8/34 H1N1 influenza strain while in most other studies the H3N2 [15, 32, 33] or A/WSN/33 H1N1 [30] influenza strain is used. Replication of RSV and PVM in the epithelium allows OVA to penetrate the barrier of the respiratory mucosa and promotes allergen sensitization [34]. Furthermore, RSV and PVM infection of epithelial cells could enhance the production of β -chemokines (a.o. MIP-1 α , eotaxin, RANTES) [35] and expression of ICAM-1 [36] by epithelial cells, which are both involved in allergic inflammation. Influenza virus is predominantly located in macrophages, which produce, upon activation, different mediators, among them the Th1 cytokines IL-12, IL-1, TNF- α and IFN- γ , promoting cell-mediated immunity [37]. The synergistic effect of these cytokines could result in a stronger Th1-mediated inhibition of the Th2 cytokine response.

Eosinophilic leukocytes play prominent roles in promoting pathophysiologic conditions such as respiratory allergy and airway hyper-reactivity [3]. Influenza virus and PVM infection increased significantly the infiltration of eosinophils in lungs of OVA-allergic mice. Thus, influenza virus infection aggravated eosinophilia despite the unaltered expression of

Th2 cytokines and eotaxin. However, Domachowske et al. demonstrated that IL-5 is not a crucial mediator of pulmonary eosinophilia in response to respiratory virus infection [20]. In addition, eosinophilic infiltration in lungs of allergic mice has been reported independently of IL-4, IL-5, and IL-13 [38], suggesting a novel mechanism for eosinophilia. Macrophage inflammatory protein-1 α (MIP-1 α) has been characterized as an eosinophil chemoattractant independent of IL-5, RANTES and eotaxin [3]. We demonstrated that pulmonary MIP-1 α levels were similar in RSV, PVM or influenza virus infected OVA-allergic and non-allergic mice, while no eosinophils were detected in lungs of infected non-allergic mice. We, therefore, conclude that MIP-1 α is likely not responsible for the enhanced pulmonary influx of eosinophils in virus-infected allergic mice. It therefore remains unknown which factor is responsible for the enhanced eosinophilic infiltration in influenza virus-infected OVA-allergic mice.

In summary, the results presented in this article show that the paramyxoviruses RSV and PVM both are able to enhance the allergy-induced Th2 immune response in BALB/c mice, in contrast to the orthomyxovirus influenza A. Despite the absence of enhanced Th2 cytokine and β -chemokine expression, influenza virus increased the influx of pulmonary eosinophils in OVA-allergic mice. The different effects of these viruses could be due to variation in the site of replication in the lung, or to differences in the capacity of viral proteins (especially the G protein) to modulate the allergic immune response.

Acknowledgements

The authors would like to thank H. Näring, M. van Eck, W. Vos, and G. van Amerongen for biotechnical assistance. They are also grateful for the (immuno)histochemical assistance of J.E. Robinson and F.K. Gielis-Proper. Dr Roholl is acknowledged for his stimulating discussions. Finally, we acknowledge Dr N. Nagelkerke for his statistical advice.

References

- 1 World Health Organization. Bronchial Asthma. WHO fact Sheet No 206; January 2000. <http://www.who.int/inf-fs/en/fact206.html>.
- 2 Savelkoul HF, Neijens HJ. Immune responses during allergic sensitization and the development of atopy. *Allergy* 2000; 55:989–97.
- 3 Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001; 344:350–62.
- 4 Murray M, Webb MS, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. *Arch Dis Child* 1992; 67:482–7.
- 5 Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med* 2000; 161:1501–7.
- 6 Collins PL, Chanock RM, Murphy BR. Respiratory Syncytial Virus. In: Fields BN et al. eds. *Fields virology*. Philadelphia: Lippincott-Raven Publishers, 2001; 1443–85.
- 7 Barends M, Boelen A, de Rond L et al. Influence of respiratory syncytial virus infection on cytokine and inflammatory responses in allergic mice. *Clin Exp Allergy* 2002; 32:463–71.

- 8 Peebles RS Jr, Sheller JR, Johnson JE, Mitchell DB, Graham BS. Respiratory syncytial virus infection prolongs methacholine-induced airway hyperresponsiveness in ovalbumin-sensitized mice. *J Med Virol* 1999; 57:186–92.
- 9 Barends M, Boelen A, de Rond L et al. Respiratory syncytial virus enhances respiratory allergy in mice despite the inhibitory effect of virus-induced interferon-gamma. *J Med Virol* 2003; 69:156–62.
- 10 van den Hoogen BG, de Jong JC, Groen J et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nature Med* 2001; 7:719–24.
- 11 Chambers P, Pringle CR, Easton AJ. Molecular cloning of pneumonia virus of mice. *J Virol* 1990; 64:1869–72.
- 12 Ling R, Pringle CR. Polypeptides of pneumonia virus of mice. I. Immunological cross-reaction and post-translational modifications. *J Gen Virol* 1989; 70:1427–40.
- 13 Murphy BR, Webster RG. Orthomyxoviruses. In: Fields BN et al. eds. *Field virology*. Philadelphia: Lippincott-Raven Publishers, 1996; 1397–445.
- 14 Suzuki S, Suzuki Y, Yamamoto N, Matsumoto Y, Shirai A, Okubo T. Influenza A virus infection increases IgE production and airway responsiveness in aerosolized antigen-exposed mice. *J Allergy Clin Immunol* 1998; 102:732–40.
- 15 O'Donnell DR, Openshaw PJ. Anaphylactic sensitization to aeroantigen during respiratory virus infection. *Clin Exp Allergy* 1998; 28:1501–8.
- 16 Teichtahl H, Buckmaster N, Pertnikovs E. The incidence of respiratory tract infection in adults requiring hospitalization for asthma. *Chest* 1997; 112:591–6.
- 17 Eriksson M, Bennet R, Nilsson A. Wheezing following lower respiratory tract infections with respiratory syncytial virus and influenza A in infancy. *Pediatr Allergy Immunol* 2000; 11:193–7.
- 18 Boelen A, Kwakkel J, Barends M, de Rond L, Dormans J, Kimman T. Effect of lack of Interleukin-4, Interleukin-12, Interleukin-18, or the Interferon-gamma receptor on virus replication, cytokine response, and lung pathology during respiratory syncytial virus infection in mice. *J Med Virol* 2002; 66:552–60.
- 19 Su Y-H, Yan X-T, Oakes JE, Lausch RN. Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection. *J Virol* 1996; 70:1277–81.
- 20 Domachowske JB, Bonville CA, Easton AJ, Rosenberg HF. Gene expression in epithelial cells in response to pneumovirus infection. *J Leukoc Biol* 2002; 71:966–72.
- 21 Byrd LG, Prince GA. Animal models of respiratory syncytial virus infection. *Clin Infect Dis* 1997; 25:1363–8.
- 22 Domachowske JB, Bonville CA, Rosenberg HF. Gene expression in epithelial cells in response to pneumovirus infection. *Respir Res* 2001; 2:225–33.
- 23 Cook PM, Eglin RP, Easton AJ. Pathogenesis of pneumovirus infections in mice: detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs of infected mice by *in situ* hybridization. *J Gen Virol* 1998; 79:2411–7.
- 24 Bonville CA, Easton AJ, Rosenberg HF, Domachowske JB. Altered pathogenesis of severe pneumovirus infection in response to combined antiviral and specific immunomodulatory agents. *J Virol* 2003; 77:1237–44.
- 25 Lamb RA, Krug RM. *Orthomyxoviridae: the viruses and their replication*. In: Fields BN, ed. *Fields virology*. Philadelphia: Lippincott-Raven Publisher, 1996; 1353–95.
- 26 Graham BS, Johnson TR, Peebles RS. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. *Immunopharmacology* 2000; 48:237–47.
- 27 Srikiatkachorn A, Braciale TJ. Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection. *J Virol* 1997; 71:678–85.
- 28 Hancock GE, Speelman DJ, Heers K, Bortell E, Smith J, Cosco C. Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus. *J Virol* 1996; 70:7783–91.
- 29 Johnson TR, Fischer JE, Graham BS. Construction and characterization of recombinant vaccinia viruses co-expressing a respiratory syncytial virus protein and a cytokine. *J Gen Virol* 2001; 82:2107–16.
- 30 Yilma T, Zee YC, Osebold JW. Immunofluorescence determination of the pathogenesis of infection with influenza virus in mice following exposure to aerosolized virus. *J Infect Dis* 1979; 139:458–64.
- 31 Miller RR. Viral infections of the respiratory tract. In: Thurlbeck WM, ed. *Pathology of the lung*. New York: Thieme medical publishers, 1988; 147–79.
- 32 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.
- 33 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.
- 34 Freiherst J, Piedra PA, Okamoto Y, Ogra PL. Effect of respiratory syncytial virus infection on the uptake of and immune response to other inhaled antigens. *Proc Soc Exp Biol Med* 1988; 188:191–7.
- 35 Welliver RC. Immunology of respiratory syncytial virus in infection: eosinophils, cytokines, chemokines and asthma. *Pediatr Infect Dis J* 2000; 19:780–3.
- 36 Wang S, Halsworth PG, Dowling KD, Alpers JH, Bowden JJ, Forsyth KD. Adhesion molecule expression on epithelial cells infected with respiratory syncytial virus. *Eur Respir J* 2000; 358–66.
- 37 Wijburg OLC, Dinatale S, Vadolas J, van Rooijen N, Strugnell RA. Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection. *J Virol* 1997; 71:9450–7.
- 38 Proust B, Nahori MA, Ruffie C, Lefort J, Vargaftig BB. Persistence of bronchopulmonary hyper-reactivity and eosinophilic lung inflammation after anti-IL-5 or -IL-13 treatment in allergic BALB/c and IL-4Ralpha knockout mice. *Clin Exp Allergy* 2003; 33:119–31.