

# Anaphylactic sensitization to aeroantigen during respiratory virus infection

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## Summary

**Background** Virus infections frequently exacerbate asthma, and in some cases may even precipitate its onset. Although this association is well known, experimental investigation has been hampered by the lack of adequate models.

**Objective** The effects of acute respiratory virus infection on sensitization to aeroallergen were investigated in this study.

**Methods** Nebulized ovalbumin was used as an aeroantigen in normal mice, and in those infected with respiratory syncytial virus or influenza A.

**Results** Both viruses caused transient illness. Ovalbumin inhalation did not induce specific serum antibodies unless the mice were infected at the time of nebulization. In exposed uninfected mice cutaneous challenge with ovalbumin caused no response, but caused acute systemic illness and collapse if previous pulmonary exposure had occurred during respiratory infection. Mice that collapsed in response to cutaneous ovalbumin were found to have IgG1 specific to ovalbumin that was not found in the other mice. Intracellular cytokine staining of splenocyte cultures showed ovalbumin-specific production of IL-4 was enhanced by virus infection during exposure. In CD8<sup>+</sup> T cells, ovalbumin-specific interferon- $\gamma$  production was also enhanced by co-infection with influenza. Both viruses were equally associated with the induction of anaphylaxis.

**Conclusion** These results show that infection with respiratory viruses powerfully augments cellular and humoral immune responses to aeroantigen and provide an experimental model that allows such effects to be investigated.

**Keywords:** allergy, influenza, anaphylaxis, respiratory syncytial virus, bronchiolitis, asthma, ovalbumen

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## Introduction

A link between respiratory virus infection in infancy and the development of asthma has been postulated for many years. Wheezing after severe infantile bronchiolitis is common and bronchial hyperresponsiveness to direct testing can be shown, even many years later [1]. Children with asthma who become sensitized to environmental allergens have been found to be more likely to continue to have symptoms [2]. Acute bronchiolitis is the commonest cause of admission to

hospital in the first year of life [3]. Sigurs *et al.* [4] showed that respiratory syncytial virus (RSV) bronchiolitis during the first year of life was an important factor in the development of asthma. Children who had severe RSV bronchiolitis were more likely to develop skin-prick sensitization and serum IgE to common food and inhaled allergens. RSV bronchiolitis was the single most important risk factor for this, and a family history of atopy or asthma further increased the risk.

Allergic asthma is characterized by airways inflammation, even in mild disease. Specifically, this inflammation includes airway wall infiltration by TH2 lymphocytes, eosinophils and mast cells [5–7]. The net effect is the production of TH2 cytokines such as IL-4 in response to

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certain antigens and switching to Ig isotypes such as IgE. IgE binds to the high affinity IgE receptor, FcεR1, on mast cells and primes them for activation by further antigen [8,9]. FcεR1 expression is pronounced on eosinophils, basophils and monocytes in atopic individuals [10]. Mast cells and eosinophils also release IL-4 and IL-5 further favouring a TH2 state [11,12].

In this study the effects of acute respiratory virus infection on sensitization of BALB/c mice to nebulized ovalbumin were examined. Using this mouse strain, ovalbumin sensitization has been shown using repeated nebulization [13]. In this model, mice develop allergic responses including airway hyperresponsiveness, allergen specific IgE and IgG1, and cutaneous hypersensitivity [13,14]. This study shows that intercurrent viral infections greatly enhance sensitization to this aeroantigen.

## Methods

### *Animals, infections and sensitization*

Eight–12-week-old female BALB/c mice were obtained from Harlan Olac (Harlan Olac, Bicester, UK). Mice were kept in pathogen free conditions throughout. All bedding, water and feed was dust-free and sterilized. Cages were covered with filter tops and kept in a filter cabinet.

Mice were lightly anaesthetized with ether and infected intranasally with  $5 \times 10^6$  plaque forming units, A2 strain RSV (86 µL), 86 µL of influenza virus (X31) or 86 µL of PBS (mock infection). X31 strain influenza virus was a kind gift from Dr Alan Douglas from the National Institute of Medical Research, Mill Hill, London, UK (4096 haemagglutination units/50 µL and it was diluted 1:100 in PBS before infection). RSV was prepared as described by Bangham *et al.* [15].

A transparent plastic container measuring 40 cm by 30 cm and 30 cm in height was used as a chamber for nebulization. Mice were placed in the container, the lid replaced and the entire container placed in a class 1 cabinet. A Ventstream nebulizer (Medic-Aid, Pagham, UK) was filled with either 1% ovalbumin in PBS or PBS alone and attached to the nebulization container. The nebulizer was driven by compressed air at a rate of 10 L/minute. Nebulization occurred for 20 min a day for 10 days, the total volume per nebulization was 12 mL of liquid. After each nebulization the containers were thoroughly cleaned and dried before reuse.

### *Study protocol*

After intranasal infection the mice were exposed daily for 20 min to nebulized 1% ovalbumin in PBS, for 10 consecutive days. Control mice had nebulization with PBS alone. The nebulization occurred either from days 4–13

after infection, days 11–20 after infection or days 18–27 after infection. Mice were weighed regularly as an indication of the severity of illness. After 10 nebulizations the mice were left for 5 days.

### *Intradermal challenge*

A sterile solution of 500 mg/mL ovalbumin in PBS was prepared. On the fifth day after the last ovalbumin challenge a  $\text{cm}^2$  area was shaved on the abdomen of the mice and an intradermal injection of 20 µL of ovalbumin solution given (total dose 10 mg). Mice primed by intraperitoneal (i.p.) injection of 200 µL alum precipitated ovalbumin either once (10 days earlier), twice (two months earlier and 10 days earlier) and unprimed mice were used as controls. The mice were observed for 2 h. The mice were given a lethal dose (3 mg) of pentobarbital i.p. and exsanguinated. The serum was kept for enzyme linked immunosorbant assay (ELISA).

### *Measurement of Ig to ovalbumin*

ELISA plates (96-well; Maxisorp, Nunc-Nalgene, Hereford, UK) were coated with 100 µL/well 0.1 mol/L  $\text{NaHCO}_3$  pH 8.2 containing 5 µg/mL of either rat anti-mouse IgE, rat anti-mouse IgG1 or rat anti-mouse IgG2a and incubated overnight at 4 °C (antibodies from Serotec, Oxford, UK). After washing three times with PBS/0.05% Tween the plates were blocked with 200 µL/well 2% BSA in PBS for 1 h at 37 °C. After washing as above, 100 µL of a 1/10 dilution of serum in 1% BSA in PBS was then added to each well and the plates incubated for 1 h at 37 °C.

Digoxigenin was conjugated to ovalbumin using a digoxigenin conjugation kit (Boehringer Mannheim, Lewes, UK). The ratio of ovalbumin:digoxigenin conjugation was 1:40. The plates were washed again as above and 100 µL/well of digoxigenin-conjugated ovalbumin was added (diluted 1:50 in 1% BSA in PBS). The plates were then incubated for 1 h at 37 °C and then washed three times as before. Horseradish peroxidase (HRP) -conjugated sheep antidigoxigenin Fabs (Boehringer Mannheim) were diluted 1:3000 in 1% BSA in PBS and 100 µL/well was then added. The plates were then incubated at 37 °C for 1 h. After washing, O-phenylenediamine (OPD) substrate was added (0.034% in citrate-phosphate buffer) and left for 15 min for colour development, the reaction was stopped with 50 µL 2 mol/L  $\text{H}_2\text{SO}_4$  and the plates read at 490 nm. Absorbance readings for different groups were compared using Mann–Whitney test for significance at 0.95.

### *In vitro splenocyte culture with ovalbumin*

Spleens were removed from the mice by careful aseptic technique and disrupted by compression through a cell

strainer (Falcon, Fisher Scientific, Loughborough, UK). They were placed in RPMI (Sigma, Poole, UK) with 10% fetal calf serum, penicillin 50 µg/mL, streptomycin 50 µg/mL (pen./strep.) and glutamine 300 µg/mL. Splenocytes ( $3 \times 10^6$ /well) were plated in 24-well tissue culture dishes (1.5 mL total volume/well) for 72 h with different concentrations of ovalbumin (4, 20, 100 and 500 µg/mL).

#### *Intracellular cytokine staining and flow cytometric analysis*

For intracellular cytokine staining splenocytes were stimulated for 4 h at 37 °C with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng/mL ionomycin (Calbiochem, Nottingham, UK). All cell incubations were performed in non-tissue culture plastic grade tubes to prevent adherence of cell populations during stimulation. Ten micrograms per millilitre brefeldin A (Sigma) was included for the last 2 h of incubation to disaggregate the Golgi complex, thereby allowing intracellular cytokines to accumulate. Once stimulated, cells were harvested, washed in isoton (azide-free balanced electrolyte solution; Coulter Electronics Ltd, Luton, UK) and fixed in 2% formaldehyde in hypertonic PBS for 20 min at room temperature.

Fixed cells were then transferred to conical tubes and stained for intracellular cytokines. The cells were first permeabilized with 0.5% saponin diluted in PBS containing 1% BSA and 0.1% sodium azide (PBS/BSA/Azide) for 10 min at room temperature. Once permeabilized, all subsequent washes and antibody dilutions were performed in this permeabilization solution at room temperature unless otherwise stated. Cell suspensions were then treated with

5 µg/mL of rat anti-IL-4 (11B11, IgG1) conjugated to fluorescein isothiocyanate (FITC) and 5 µg/mL of phycoerythrin (PE)-conjugated rat anti-mouse IFN-γ (AN18 PE) and incubated for 30 min at room temperature. After 2 washes, saponin was removed by washing with PBS/BSA/Azide to close permeabilized cells before the addition of the appropriate dilutions of either quantum red-conjugated rat anti-mouse CD4 (IgG2a, Sigma), rat anti-mouse CD8 (IgG2a, Sigma) or isotype matched control (to define background for flow cytometric (FCM) analysis). Conjugated anti-cytokine antibodies were a kind gift of Dr Anne O'Garra (DNAX, Palo Alto, CA, USA). FCM analysis was performed using an Epics ELITE flow cytometer/cell sorter (Coulter, Luton, UK).

#### **Results**

##### *Effect of ovalbumin nebulization on weight loss during viral infection*

Mice exposed to ovalbumin nebulization lost and regained the same amount of weight as controls (PBS alone). Weight loss followed the same time course in mice exposed to ovalbumin as those given PBS alone (Fig. 1). In different experiments there was some variation in the percentage of weight lost in RSV-infected mice, being sometimes greater or less than the percentage seen after influenza infection. However, the timing of weight loss and recovery was consistent: influenza-challenged mice lost weight from day 2, whereas RSV-infected mice lost weight after day 4 post-infection. Peak weight loss occurred at day 6 or 7 after infection, followed by steady recovery.

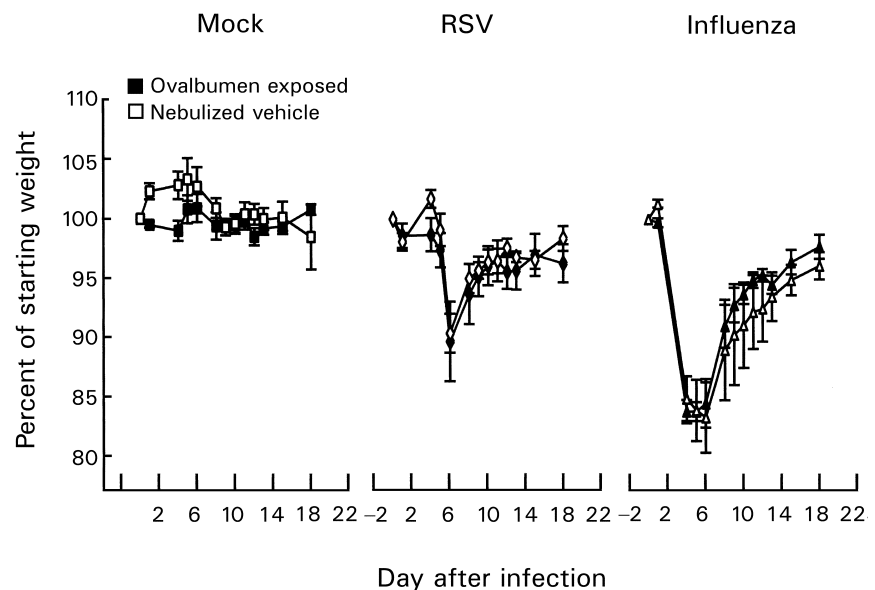


**Fig. 1.** Weight loss in experimental groups. Mock infected mice did not lose weight whether they were exposed to ovalbumin or nebulized vehicle alone from days 4–10 inclusive. Mice infected with RSV or influenza A lost weight, but the weight loss was unaffected by ovalbumin nebulization. In other studies, similar administration of RSV caused greater weight loss than influenza A (not shown).  $n = 6$  for ovalbumin exposed mice and  $n = 5$  for PBS-exposed mice for the experiment shown.

*Acute anaphylactic shock on intradermal infection*

Intradermal injection of ovalbumin caused acute collapse in mice given inhaled ovalbumin during acute respiratory virus infection (Table 1). The mice became immobile and appeared unwell. The mucosae and nose became cyanosed rather than the normal pink appearance and the respiratory effort increased. Bleeding by transection of the femoral artery was found to be impossible during this period because of cardiovascular shut down. These changes occurred 15–20 min after injection. Mice allowed to recover from this anaphylactic collapse appeared normal 2 h later. Acute collapse was only seen in mice given ovalbumin nebulization after virus infection and not in mock infected animals; it did not occur in virus-infected animals exposed to PBS nebulization instead of ovalbumin. Acute collapse was not seen in mice challenged with ovalbumin on days 11–20 or days 18–27 after virus infection. Anaphylactic collapse was seen in every animal tested, when primed by virus infection and ovalbumin nebulization from days 4–13. Control animals given i.p. alum precipitated ovalbumin (alum/ova.) either once or twice (as above) also became immobile and unwell on intradermal ovalbumin challenge given 10 days after the last alum/ova dose.

Virus infection enhances TH2 cytokine production by splenocytes from mice exposed to ovalbumin. Intracellular cytokine staining of cells demonstrated higher IL-4 production in splenocytes from mice infected with influenza and RSV than mock infected (Fig. 2). Interestingly a higher proportion of CD8 cells were positive for IL-4 than CD4

cells. The influenza virus infected mice had more IL-4-producing cells than RSV-infected mice where the difference from mock infected mice was less marked. When the percentage positive was compared with the dose of ovalbumin in the culture, the differences become clearer. A dose response effect on cytokine production is seen in CD8<sup>+</sup> cells but not in CD4<sup>+</sup> cells: at higher concentrations of ovalbumin more CD8 cells are positive for both IL-4 and IFN- $\gamma$ . In all cultures the production of IL-4 by CD8<sup>+</sup> cells was higher than IFN- $\gamma$ . However, only in influenza-infected mice did CD4<sup>+</sup> cells produce more IL-4 than IFN- $\gamma$ .

Exposure to ovalbumin during acute respiratory virus infection leads to ovalbumin-specific IgG1 production. An IgG1 response to ovalbumin was detected in mice sensitized by ovalbumin nebulization, only if they also had an acute viral respiratory infection (Fig. 3). Mice given alum precipitated ovalbumin i.p. 10 days earlier were used as a positive control. Mice exposed to ovalbumin nebulization after mock infection had no detectable IgG1 response to ovalbumin. No significant IgG2a against ovalbumin was detectable except for control mice given alum-precipitated ovalbumin i.p. 10 days earlier.

Specific IgG1 response to ovalbumin was only seen when ovalbumin nebulization was performed during the period of acute weight loss (days 4–13). Mice given RSV or influenza, and then exposed to nebulized ovalbumin during the period of recovery (days 11–20) or after weight was regained (days 18–27) had no detectable IgG1 to ovalbumin (Fig. 4). Influenza infection was associated with higher levels of IgG1 to ovalbumin than RSV infection. This did not appear to reflect the severity of illness, since mice lost more weight after RSV infection than after influenza.

**Table 1.** Collapse after cutaneous ovalbumin in sensitized mice

|           | Pulmonary challenge |           |     |
|-----------|---------------------|-----------|-----|
|           | RSV                 | Influenza | PBS |
| Ovalbumin | +                   | +         | +   |
| PBS       | –                   | –         | –   |

Mice exposed to ovalbumin nebulization during acute RSV or influenza (from day 4 for 10 days) collapsed when challenged with intradermal ovalbumin 18 days after they were infected. Mice infected but not exposed to ovalbumin nebulization and uninfected mice did not collapse on intradermal ovalbumin challenge. Mice exposed to ovalbumin during convalescence (from day 11 for 10 days) or after recovery (from day 18 for 10 days) did not collapse on intradermal ovalbumin challenge. Mouse response to challenge was rated on a scale of – (no response) to + + + (moribund). The data are representative of two separate experiments with six mice/group for ovalbumin exposed and five mice/group PBS exposed. + + + indicates that all the mice in the group became severely ill. – indicates that no mice in the group appeared ill in any way.

**Discussion**

A role for respiratory viruses in predisposing to asthma is an attractive but controversial hypothesis [16,17]. Recently it has been suggested that virus exposure in early life has at most a minor role in the aetiology of wheezing and may in fact be protective against both allergies and asthma [18]. Martinez has argued that acute respiratory viruses produce predominantly TH1 responses and may inhibit the development of TH2 cells that are associated with allergy [2]. This study has shown that mice can be sensitized to ovalbumin without the need for adjuvants if they have an acute respiratory viral infection. Indeed intradermal ovalbumin challenge in such mice was associated with acute anaphylaxis. Acute anaphylactic shock in mice is accompanied by marked hypoactivity that can be reversed with opiate antagonists [19]. It can be mediated through IgE or IgG1, both of which bind to mast cells in the mouse. In some strains (CTS, DS, C57BL/6) anaphylaxis is far less common in younger mice (1.5–2.5 months). In other strains, such as BALB/c, DBA/2

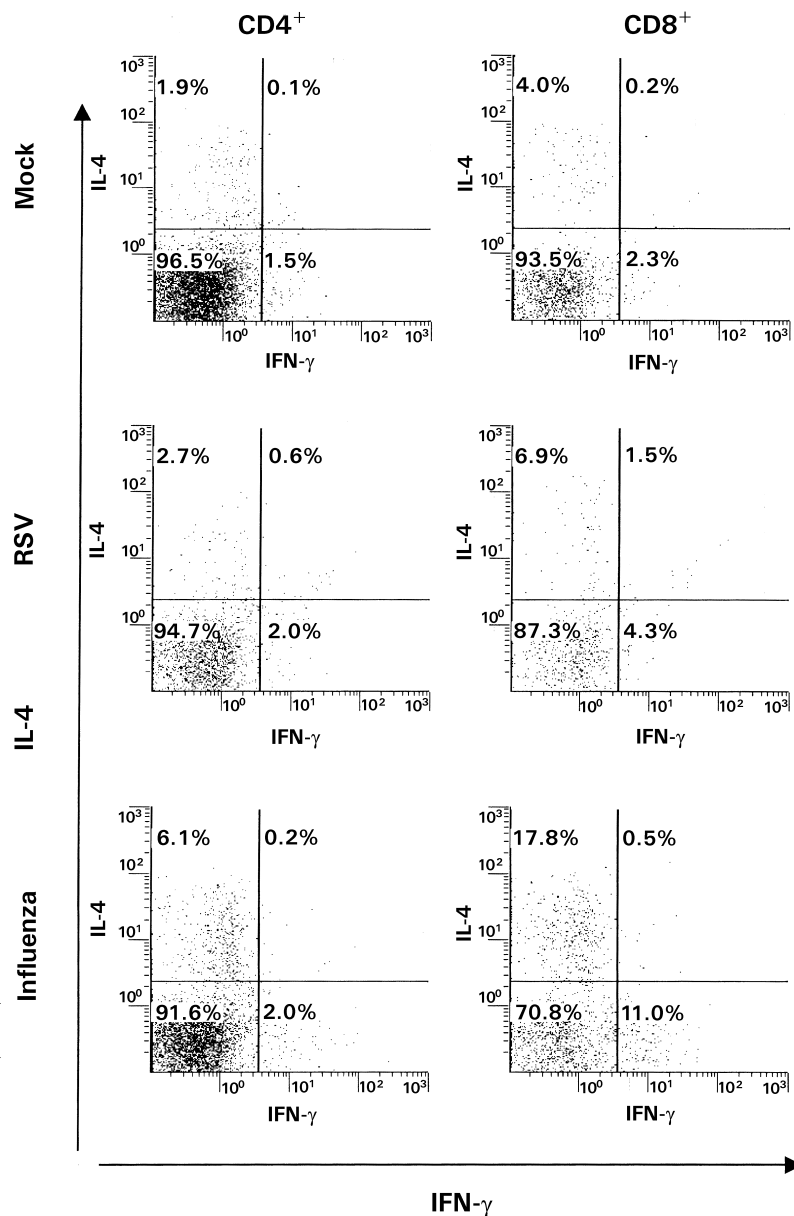


**Fig. 2.** Flow cytometric analysis of IL-4 and interferon- $\gamma$  production in spleen cells cultured with ovalbumin. Spleen cells from mock infected mice, mice infected with RSV or influenza were exposed to 500  $\mu\text{g/ml}$  of ovalbumin *in vitro*. At 72 h, cells were stained for CD4 or CD8, fixed permeabilized and stained for intracellular IL-4 and interferon- $\gamma$ . Spleen cells from control mice showed only low levels of production of either cytokine. Ovalbumin exposure during infection with RSV caused a small but appreciable number of antigen-specific cytokine-producing T cells to appear. This augmentation was greater in mice infected with influenza A. The data reflect the pooled spleen cultures from three separate mice.

and C3H, anaphylaxis can be produced at all ages [20]. The main mediator for anaphylactic shock in mice is platelet-activating factor released from mast cells [21]. IgG1 is thought to be more effective than IgE in producing systemic sensitization in some strains [22]. As far as the authors are aware, this mouse model is the first to produce acute anaphylactic shock to an allergen, without the use of adjuvants.

Recent data indicates that mice can be sensitized to ovalbumin and other protein antigens via the respiratory route [14]. By this method of sensitization, mice develop allergic responses including allergen specific IgE, IgG1, cutaneous hypersensitivity and airway hyperresponsiveness

[13]. Others have shown that isotype switching in the mouse occurs from IgM to IgG1 and then IgE [23]. In the work of Schwarze *et al.* [24], RSV infection enhanced airways hyperresponsiveness but did not enhance ovalbumen specific IgE or IgG1 levels. This result is consistent with data from this study, which show that IgG1 production and anaphylaxis could only be demonstrated if exposure to ovalbumen occurred during the peak of illness.

Previous authors have suggested that respiratory virus infection may have a role in augmenting allergic responses. Previous studies have exposed mice to alum precipitated ovalbumin (alum/ova) during influenza infection [25,26];

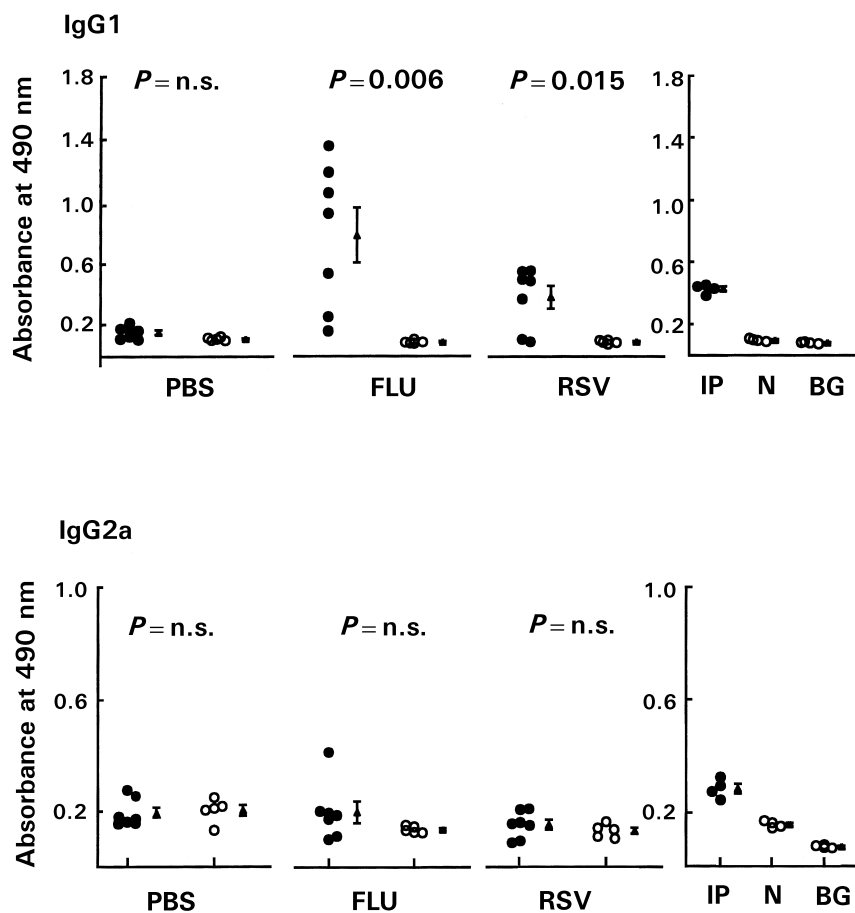


**Fig. 3.** Ovalbumin-specific production of IgG1. Ovalbumin nebulization (filled symbols) during infection with influenza or RSV caused high levels of ovalbumin-specific IgG1 to be generated (centre panels). Ovalbumin-specific IgG1 levels were comparable with or in excess of those caused by i.p. injection of alum precipitated ovalbumin (far right panel). Ovalbumin nebulization did not produce a detectable IgG1 response in the absence of infection (far left panel). Nebulized vehicle alone (open symbols) was not associated with specific IgG1 production. (N is normal mouse serum, BG is ELISA background). Although i.p. injection of ovalbumin precipitated with alum generated specific IgG2a and IgE responses, no such responses occurred in mice exposed to inhaled ovalbumin, regardless of virus infection (not shown).

and during RSV infection [27], suggesting an augmentation of the allergic response to ovalbumin seen with alum/ova alone. In each case no direct measurement of IgE or IgG1 was made but passive cutaneous anaphylaxis (PCA) was used as an indirect measure of sensitization. Freihorst *et al.* also used ovalbumin sensitization during RSV infection with and without alum [28]. IgE was again assessed by PCA and no IgE was found without the use of adjuvant. Acute anaphylactic shock was not seen. Kudlacz and Knippenberg recently showed that sensitization of guinea-pigs to ovalbumin during parainfluenza virus infection diminishes lung histamine release on re-challenge with ovalbumin [29]. However, their findings and interpretation may not conflict with data from this study. Firstly, although acute RSV infection is associated with a vigorous TH1 antiviral response, individual RSV proteins may induce TH2 responses [30]. Alwan *et al.* have shown that immunity to the G protein of RS virus is associated with TH2 responses when splenocytes from primed mice are re-exposed *in vitro* to RSV [31]. Secondly, the timing of antigen exposure may be very important. Levels of environmental antigens, such as house dust mite, moulds and pollens, are likely to be at a

constant level throughout the acute phase when infants have a primary infection. In the work of Kudlacz *et al.* for example, ovalbumin exposure was limited to 2 days separated by a week and commenced on day 7 or day 19 after infection and when parainfluenza antigen was no longer detectable in the lung [29]. This may have had a very different effect on the ovalbumin response. In this studies, exposure to ovalbumin was not associated with sensitization if it occurred during the recovery or convalescent phases of acute respiratory viral infection.

Primary sensitization to an allergen would be unlikely through an intact mucosal epithelium. The normal respiratory mucosal surface has evolved in an environment rich in airborne pollens, grasses and other antigens. However, inflammation of the respiratory mucosal epithelial surface may allow the entry of allergens, or the exposure of mast cells, B cells and T cells to allergens in a cytokine-rich environment and lead to sensitization. Respiratory virus infections are common and can cause severe airways inflammation. They are especially common in childhood. RSV outranks all other respiratory infections as a cause of severe respiratory illness in young children during a time

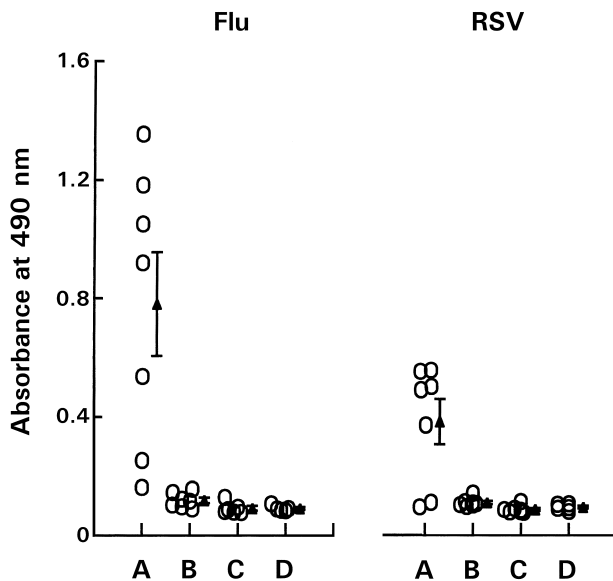


**Fig. 4.** Ovalbumin-specific IgG1 induction according to the time of sensitization. Mice exposed to ovalbumin after infection with influenza (left panel) or RS virus developed strong IgG1 responses to ovalbumin only if nebulization was performed from day 4 for 10 days (A) but not if administered from day 11 for 10 days (B), from day 18 for 10 days or if exposed to PBS nebulization alone (D).

when their relatively immature immune system is exposed to a wide range of antigens for the first time.

Mucosal surfaces such as the gut and lung are continuously exposed to soluble antigens. They have a role in preventing harmful immune responses to non-pathogenic substances that do reach the body interior as well as vigilance against pathogens [32]. Tolerance has been linked to the expression of immunosuppressive cytokines such as IL-4, IL-10 and TGF- $\beta$  [33]. It is striking that the cytokines that are involved in the generation of tolerance and the amelioration of disease are also involved in allergic responses. In the context of an inflamed mucosal surface these immunosuppressive cytokines may contribute to the development of allergy.

Overall, these results strongly suggest a role for acute respiratory viral infection in the induction of TH2 responses to allergens. The finding of acute anaphylactic shock after sensitization was dramatic and unexpected. Both T- and B-cell responses to ovalbumin were generated without the use of adjuvants. This model offers novel insights into the immunopathology associated with respiratory virus infections and defines further the links with allergy and asthma.

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