

Adenoviral infection inhibits allergic airways inflammation in mice

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Summary

Background Recent epidemiological studies have suggested that exposure to certain viruses and bacteria influences the development of allergy and allergic diseases, such as asthma. However, there is a paucity of experimental evidence examining the consequences of concurrent exposure to allergen and infectious agents, and the potential mechanisms by which allergic disease might be averted as a result.

Objective To model this situation experimentally, we investigated whether a virally induced immune response, elicited by a replication-deficient human type 5 adenovirus (RDA) administered at a site distant from the airways, could inhibit ovalbumin (OVA)-induced airways eosinophilic inflammation.

Methods C57BL/6 mice were infected intramuscularly with RDA 16 h prior to intraperitoneal OVA sensitization. Cellular and cytokine responses in the lung/airways were examined after an OVA aerosol challenge.

Results RDA infection significantly inhibited the inflammatory response in the lung tissue after antigen challenge. In the bronchoalveolar lavage (BAL), total cell number, eosinophils and lymphocytes were decreased by 70, 85 and 65%, respectively, after antigen challenge in RDA-treated, compared with untreated, mice. RDA infection had no effect on IgE synthesis. The levels of IL-5, IL-4 and IFN γ in the BAL after antigen challenge were significantly lower in RDA-treated mice. *In vitro* production of cytokines by splenocytes in response to OVA restimulation revealed a shift from IL-4 in sensitized, PBS-treated mice, to IFN γ in sensitized mice treated with RDA. Flow cytometric analysis revealed that RDA infection increased the proportion of CD8 T cells in the BAL; this change in T-cell subsets was accompanied by an increase in both CD4 and CD8 T cells positive for intracellular IFN γ . Inhibition of antigen-induced airways inflammation was IFN γ -dependent but did not require IL-12, as RDA-treatment inhibited airways inflammation in IL-12 but not IFN γ knock-out mice.

Conclusion This study demonstrates that an immune response against a replication-deficient adenovirus during the initial exposure to OVA inhibits the development of airways inflammation after antigen aerosol challenge.

Keywords: mice, ovalbumin, airways inflammation, adenovirus, IgE, cytokines, eosinophils, flow cytometry

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Introduction

The prevalence of atopy, allergic rhinitis and asthma has been increasing over the last two decades in developed countries [1]. Since offspring of atopic parents are at a

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higher risk of developing asthma [2], a genome-wide search for genetic loci conferring susceptibility to asthma is actively being pursued [3,4]. However, as it is unlikely that the genetic composition of stable populations could have changed so significantly within 20 years, the contribution of additional factors to this increase should be examined. While some infections, notably respiratory syncytial virus (RSV) and rhinovirus, may precipitate the development of allergy [5,6], a number of epidemiological studies have shown that exposure to certain other infectious agents, especially in early childhood, is associated with a decreased prevalence of allergy and asthma later in life. For example, Shirakawa *et al.* demonstrated an inverse association between tuberculin responses and atopic disorders in Japanese children [7], and Matricardi *et al.* found that Italian military students with antibodies against hepatitis A virus, likely a marker of predominantly childhood infection, have a lower incidence of atopy, rhinitis and asthma [8]. Furthermore, Shaheen *et al.* showed a large reduction in allergy to house dust mite in African children who had contracted measles during an epidemic [9]. Finally, a number of studies have revealed an inverse correlation between the number of elder siblings and the incidence of atopy and allergic disease, prompting the hypothesis that older children transmit to their younger siblings infections that may protect against the development of allergy [10–13]. At present, with a paucity of direct experimental evidence, whether there is a causal relationship between these two phenomena remains speculative.

A central pathogenetic feature of asthma is the presence of a distinct immune-inflammatory reaction in the airways, with mast cells and eosinophils playing important effector roles [14–16]. It is now recognized that this reaction is driven by a polarized TH2 immune response [17,18]. Indeed, interleukin 4 (IL-4) is required to elicit events such as IgE synthesis, which is important in the 'arming' of IgE-bearing cells; IL-5 mediates eosinophil differentiation in the bone marrow and activation in target tissues. In contrast, infectious agents such as some viruses and intracellular bacteria are able to induce TH1-polarized immune responses, characterized by enhanced expression of IL-12 and IFN γ [19–21]. Based on the evidence suggesting counter-regulation between TH1 and TH2 cytokines [22,23], it has been hypothesized that early childhood exposure to aeroallergens in the course of ongoing TH1-polarized immune responses to infectious agents may influence the development of TH2-driven allergic sensitization and therefore of TH2-type immune-inflammatory reactions, such as asthma, later in life [7–13]. A murine model of antigen-induced T-cell-driven airways eosinophilic inflammation [24–26] was used to test this hypothesis experimentally.

Mice were infected intramuscularly (i.m.), i.e. at a site distant from the airways, with a human type 5 E1-deleted

replication-deficient adenovirus (RDA) [27] prior to sensitization with the OVA antigen, and airways responses were examined after an aerosol challenge with OVA. The data presented here demonstrate that eosinophilic airways inflammation was significantly reduced in mice treated with RDA prior to sensitization but had no effect on the OVA-specific IgE response. The inhibition of airways inflammation was IFN γ -dependent and IL-12-independent. Thus, this study provides experimental evidence that antigen-induced airways inflammation can be inhibited when adenoviral infection occurs concurrently with the initial antigen exposure.

Materials and methods

Animals

Female C57BL/6 mice were purchased from Harlan (Indianapolis, IN, USA); IFN γ knock-out (KO) mice (C57BL/6-*Ifgtm1Ts*) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IL-12 KO mice were kindly provided by Dr Jeanne Magram (Hoffmann La Roche, Nutley, NJ, USA) and bred in-house. All mice were maintained under specific pathogen-free conditions following a 12-h light–dark cycle. Of particular importance, mice were housed in a level B facility in which all cages, food and bedding are autoclaved. Experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Sensitization and antigen challenge protocols

Six–8-week-old mice (Harlan, Indianapolis, IN, USA) were sensitized and challenged according to a previously described protocol [26]. Briefly, mice were sensitized to OVA by two intraperitoneal injections of 8 μ g OVA (Grade V, Sigma Chemical Co., St. Louis, MO, USA) adsorbed to 4 mg aluminium hydroxide (alum.; Aldrich Chemical Company, Inc., Milwaukee, WI, USA) in phosphate-buffered saline (PBS) given 5 days apart. Mock sensitization consisted of injection of alum. in PBS only. Seven days after the second sensitization, mock and OVA-sensitized mice were placed in a Plexiglas chamber and exposed to aerosolized OVA (1% W/V in 0.9% saline) for 1 h on two occasions 4 h apart. Aerosolized OVA was produced by a Bennet nebulizer at a flow rate of 10 L/min.

Adenovirus infection

Sixteen hours prior to sensitization, mice were infected i.m. with either 6×10^8 p.f.u. of a human type 5 E1-deleted replication-deficient adenovirus (RDA) [27] or vehicle (PBS) as a control. A volume of 25 μ L was injected i.m. in each of four sites on the hind legs for a total injected volume of 100 μ L.

Collection and measurement of specimens

At specific time points after antigen challenge, mice were sacrificed and bronchoalveolar lavage (BAL) was performed as previously described [26]. The lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with PBS (0.25 mL followed by 0.20 mL); ≈ 0.3 mL of the instilled fluid was consistently recovered. Total cell counts were determined with a haemocytometer. After centrifugation, supernatants were stored at -20°C for cytokine measurements by ELISA, and cell pellets were resuspended in PBS and used to prepare smears by cytocentrifugation (Shandon Inc., Pittsburgh, PA, USA) at 300 r.p.m. for 2 min. All smears were stained with Diff-Quik stain (Baxter, McGraw Park, IL, USA). Differential cell counts in BAL were determined from at least 500 leucocytes using standard haemocytological criteria to classify the cells as either neutrophils, eosinophils, lymphocytes, or macrophage/monocytes. Blood was collected by retro-orbital bleeding at various time points after antigen challenge. Serum was obtained by centrifugation after incubating blood for 30 min at 37°C . The lung tissue was fixed in 10% formalin and embedded in paraffin. Three micrometer-thick sections were stained with haematoxylin and eosin (H & E) or Congo red to further identify eosinophils.

Splenocyte culture

Spleens were harvested 6 days after the second OVA sensitization. Collagenase II (100 U/mL) (Worthington Biochemical Corporation, NJ, USA) in Hanks' balanced salt solution (HBSS) (Gibco, Burlington, ON, USA) was injected into the organ, and pieces were incubated in collagenase II (400 U/mL) for 7 min at 37°C . Tissue was ground between the ends of sterile frosted slides and the resulting cell suspension was filtered through nylon mesh (BSH Thompson, Scarborough, Ont., Canada). Red blood cells were lysed with ACK lysis buffer, and the splenocytes were washed twice with HBSS and resuspended in RPMI supplemented with 10% FCS (Gibco), L-glutamine, and penicillin/streptomycin. Cells were plated at 8×10^5 cells/well in a flat-bottom 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) in the presence of medium alone, or with $40 \mu\text{g}$ OVA/well. After 5 days of culture, supernatants were harvested for cytokine measurement.

Cytokine and immunoglobulin measurement

ELISA kits for murine IL-4 and IFN γ were purchased from R & D Systems (Minneapolis, MN, USA) and for murine IL-5 from Amersham (Amersham, UK). The sensitivity of these kits is 5 pg/mL . Serum levels of OVA-specific mouse IgE were measured 3 days after antigen challenge using a

previously described antigen capture ELISA method [26]. Briefly, $50 \mu\text{L}$ of $5 \mu\text{g/mL}$ monoclonal antibody against mouse IgE (MoME-3; Université de Louvain, Louvain, Belgium) in 0.2 mol/L borate-buffered saline at pH 8.3 was incubated in ELISA plates (Maxisorb F96, Nunc) for 16–18 h at 4°C . Subsequently wells were blocked with 1% W/V bovine serum albumin (BSA) in PBS for 2 h at room temperature, followed by washing. Fifty microlitres of mouse serum diluted 1:5 with 0.3% W/V BSA in PBS was added to each well, incubated for 16–18 h at 4°C , and plates washed extensively. Fifty microlitres of biotin-conjugated OVA ($1 \mu\text{g/mL}$ 0.3% BSA in PBS) was added to each well and incubated for 2 h at room temperature. After washing, a 1:1000 dilution of alkaline phosphatase-conjugated streptavidin (Intermedico, Mississauga, Ont., Canada) was added, incubated, and washed. Remaining plate-bound alkaline-phosphatase activity was detected by the addition of alkaline phosphatase substrate tablets (Sigma), and optical density measured with an ELISA reader at 492 nm. Units of OVA-specific IgE were calculated according to a standard curve constructed by serial dilutions of biotin-conjugated IgE.

Flow cytometric analysis of T-cell subsets and intracellular IFN γ

BAL was recovered and cells were stained with anti-CD3 (clone 145–2C11, PharMingen Canada, Mississauga, Ont., Canada), anti-CD4 (clone H129.19, Gibco) and anti-CD8 (clone 53–6.7, PharMingen). Five thousand events were collected within the lymphocyte gate (FSC vs SSC). Intracellular cytokine expression was determined according to a standard protocol [28]. Briefly, after overnight culture in 20 ng/mL PMA (Sigma) and $2 \mu\text{mol/L}$ ionomycin (Sigma), cells were incubated in $10 \mu\text{g/mL}$ Brefeldin A (Sigma) for 4 h. Subsequently, cells were stained for CD4 and CD8, and for intracellular IFN γ using an anti-IFN γ mAb (clone XMGI.2, PharMingen).

Data analysis

Data are expressed as mean $\pm$ SEM. Wherever suitable, results were interpreted using Student's *t*-test or ANOVA. The difference was considered statistically significant when $P < 0.05$.

Results

OVA challenge of OVA-sensitized mice induced an eight-fold increase in the total number of cells in the BAL relative to mock-sensitized mice challenged with OVA, with increases in monocyte/macrophages, neutrophils, lymphocytes and eosinophils. As shown in Fig. 1, RDA but not

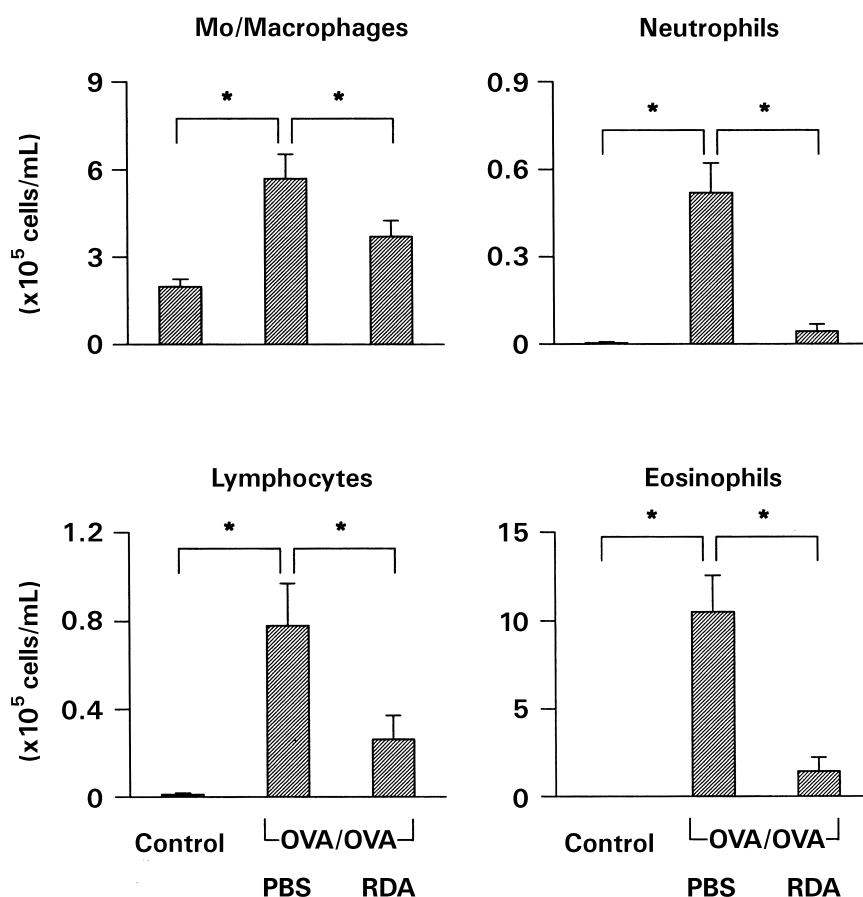


Fig. 1. RDA-mediated inhibition of antigen-induced airways inflammation. Mice were either mock- (control) or OVA-sensitized and OVA challenged. Mice received an i.m. injection of either PBS or RDA (6×10^8 p.f.u.) 16 h prior to sensitization. Data show macrophages/monocytes, neutrophils, lymphocytes, and eosinophils in BAL obtained 72 h after OVA aerosol challenge (mean $\pm$ SEM; $n = 6-9$; statistical analysis was performed using ANOVA with post hoc Fisher PLSD; *, $P < 0.05$).

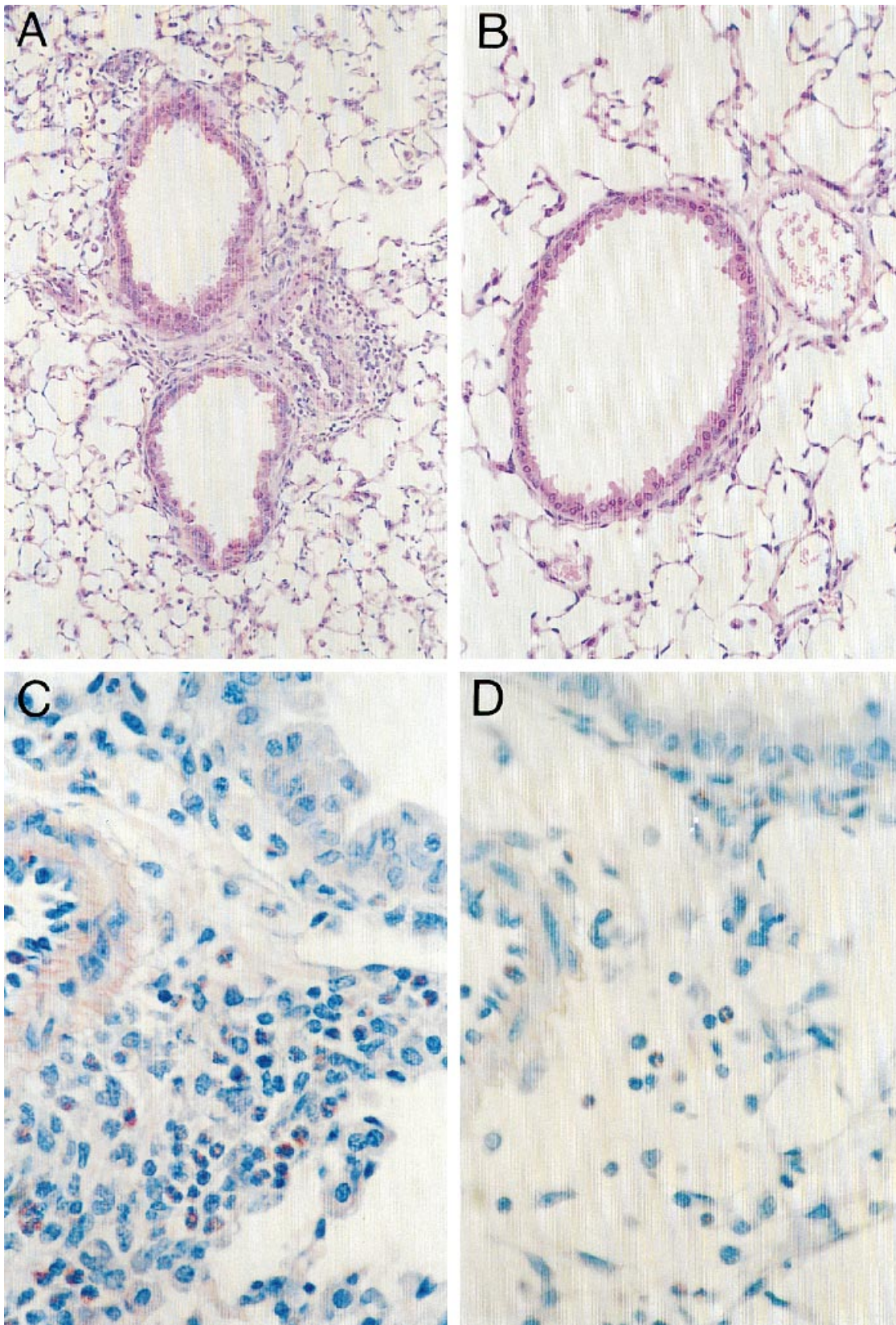
vehicle treatment significantly inhibited this airways inflammatory response. With regard to eosinophils specifically, RDA treatment resulted in a 85% reduction in the absolute number of eosinophils in the BAL. Of interest, a similar degree of inhibition was observed when using an E3-deleted or a wild-type adenovirus (data not shown). Notably, the BAL profile of RDA-treated mice was not statistically distinguishable from that of mock-sensitized controls. Changes in the BAL were confirmed by histology. Figure 2 shows lung tissues of sensitized mice 72 h after antigen challenge. A marked peribronchial and perivascular inflammatory infiltrate can be observed in lung sections of mice that received i.m. vehicle treatment prior to sensitization (Fig. 2a). In contrast, lung sections of RDA-treated mice reveal inflammation of only a mild degree (Fig. 2b). Congo red stain demonstrates a substantial proportion of eosino-

phils within the inflammatory infiltrate in sensitized and challenged mice treated with PBS only (Fig. 2c). A minimal number of eosinophils were detected in RDA-treated mice (Fig. 2d).

The effect of RDA infection prior to sensitization on IgE synthesis was then assessed. OVA-specific IgE was undetectable in mock-sensitized mice. OVA-sensitized mice that had been treated with PBS i.m. were found to have 39.2 ± 10.7 U/mL ($n = 8$) of OVA-specific IgE. RDA treatment i.m. had no significant effect on IgE synthesis, as similar levels of OVA-specific IgE were detected in these mice (29.3 ± 10.3 U/mL; $n = 8$).

Next, IL-4, IL-5 and IFN γ content in BAL obtained 24 h after antigen aerosol challenge were measured. None of these cytokines was detected in mock-sensitized, OVA-challenged mice. As shown in Fig. 3, IL-4, IL-5 and IFN γ

Fig. 2. Light photomicrograph of paraffin-embedded sections of lung tissues. All tissues were obtained 72 h after antigen challenge from OVA sensitized mice treated i.m. with either PBS [(A) and (C)] or RDA [(B) and (D)] 16 h prior to sensitization. (A) and (B) represent sections stained with haematoxylin-eosin, (C) and (D) represent sections stained with Congo red to further identify eosinophils. Note that extensive peribronchial and perivascular inflammation is observed in (A); (C) illustrates that many of these inflammatory cells are eosinophils (brownish colour). Minimal inflammation is observed in (B) and (D). Magnification: (A) 160 $\times$; (B) 250 $\times$; (C) and (D) 640 $\times$.



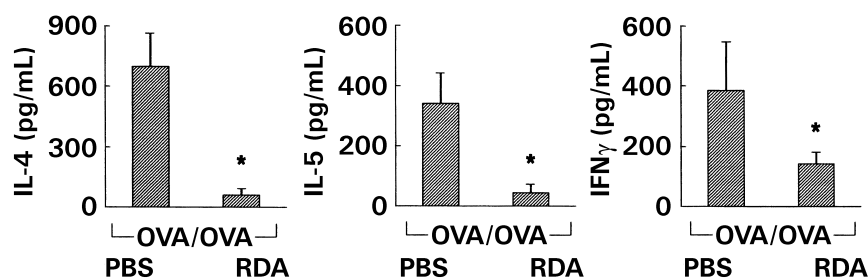


Fig. 3. Levels of IL-4, IL-5 and IFN γ in BAL supernatant. Samples were obtained 24 h after OVA aerosol challenge of OVA-sensitized mice that had received i.m. injections of either PBS or RDA (6×10^8 p.f.u.) 16 h prior to sensitization. (mean $\pm$ SEM; $n = 5$; *, $P < 0.05$ unpaired t -test).

were readily detected in the BAL of OVA-sensitized and challenged mice that had been treated i.m. with PBS. BAL cytokine content was significantly decreased in OVA-sensitized and challenged mice that had been treated i.m. with RDA.

We then assessed the *in vitro* recall response to OVA of splenocytes obtained from OVA-sensitized mice (Fig. 4). The IL-4 response was reduced by 50% in mice that had received RDA i.m. relative to mice treated with vehicle only. In contrast, the IFN γ response increased fivefold in RDA-treated mice. IL-4 and IFN γ in supernatants of splenocytes isolated from OVA-sensitized mice cultured with medium alone were below the detection limits of the assays (5 pg/mL).

RDA infection prior to sensitization significantly reduced the number of lymphocytes in the BAL after antigen challenge by $\approx 60\%$. To determine whether RDA infection altered T-cell subsets infiltrating the airways, BAL cells from OVA-sensitized mice treated with either RDA or vehicle were obtained three days after aerosol challenge and examined by flow cytometry. Table 1 shows that RDA infection prior to sensitization, while decreasing the proportion of CD4 $^+$ T cells, increased the proportion of CD8 $^+$ T cells by approximately twofold. Intracellular cytokine

analysis by flow cytometry demonstrated a three- to fivefold increase in the proportion of IFN γ^+ infiltrating T cells, both CD4 $^+$ and CD8 $^+$, in RDA-treated mice compared with mice that had received vehicle only. RDA infection therefore altered both the profile of the infiltrating T-cell subsets and the proportion of T cells expressing IFN γ .

To further investigate the role of IFN γ in RDA-mediated inhibition of antigen-induced airways inflammation, IFN γ KO mice were injected i.m. with RDA or PBS 16 h prior to OVA sensitization. After antigen challenge, a similar degree of airways inflammation in OVA-sensitized IFN γ KO mice was observed, whether treated with vehicle or RDA (Fig. 5). No statistically significant differences were observed in total cell number or specific cell types. These data suggest that RDA-mediated inhibition of airways inflammation is IFN γ -dependent. This inhibition is, however, independent of IL-12 as RDA infection prior to OVA sensitization of IL-12 KO mice (C57BL/6 p40 $-/-$) inhibited antigen-induced airways inflammation to an extent similar to that observed in wild-type mice (Fig. 6).

Discussion

It is known that respiratory infections with certain viruses,

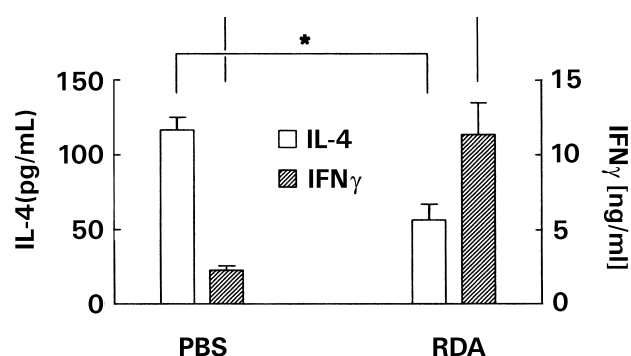


Fig. 4. *In vitro* antigen-specific IL-4 and IFN γ production by splenocytes. Splenocytes were obtained from OVA-sensitized mice treated with either PBS or RDA (6×10^8 p.f.u.) i.m. prior to sensitization. Cells were stimulated with OVA *in vitro*. (mean $\pm$ SEM; $n = 3$; statistical analysis was performed using ANOVA with post hoc Fisher PLSD; *, $P < 0.05$).

Table 1. Flow cytometric analysis of T cell subsets and intracellular IFN γ in BAL cells after antigen challenge

		Treatment*	
		PBS (%)	RDA (%)
CD4 $^+$		45.1	33.9
	IFN γ^+	4.5	12.8
CD8 $^+$		18.6	38.9
	IFN γ^+	3.5	19.9

*16 h prior to sensitization mice were infected intramuscularly with 6×10^8 p.f.u. of RDA. 72 h after OVA aerosol challenge, BAL was recovered and cells were stained for flow cytometric analysis. Data represent BAL cells pooled from eight mice.

Fig. 5. RDA-mediated inhibition of antigen-induced airways inflammation in IFN γ KO mice. Prior to sensitization, IFN γ KO mice were treated with either PBS or RDA (6×10^8 p.f.u.) i.m. Data show changes in inflammatory cells in the BAL 72 h after OVA aerosol challenge (mean $\pm$ SEM, $n = 6$; statistical analysis was performed using ANOVA with post hoc Fisher PLSD; *, $P < 0.05$).

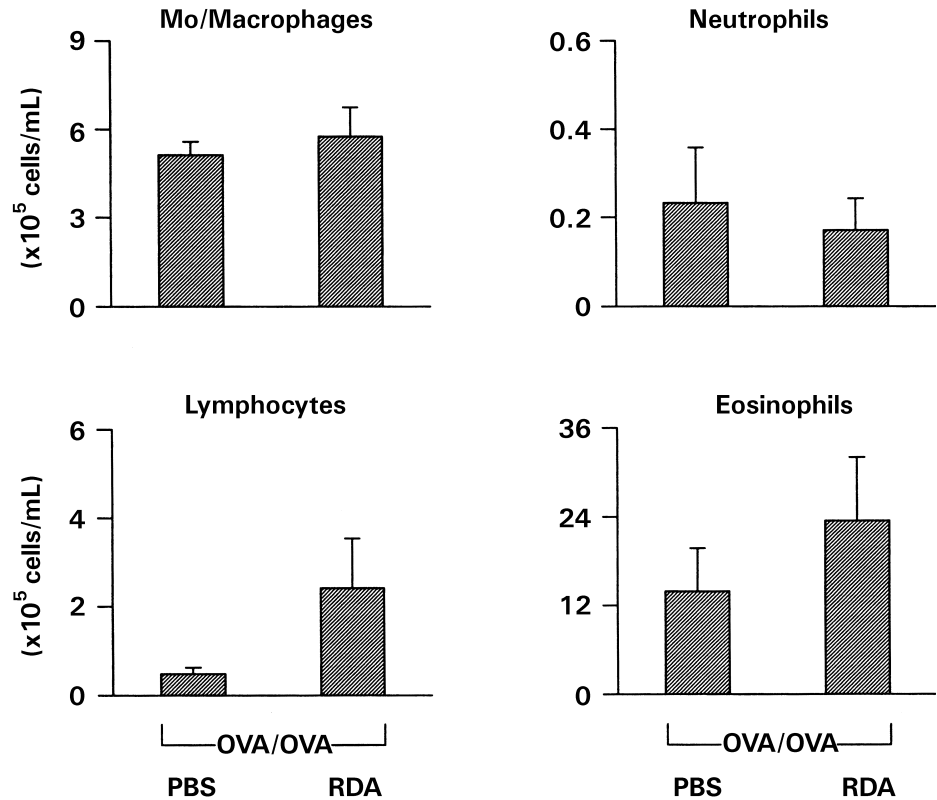
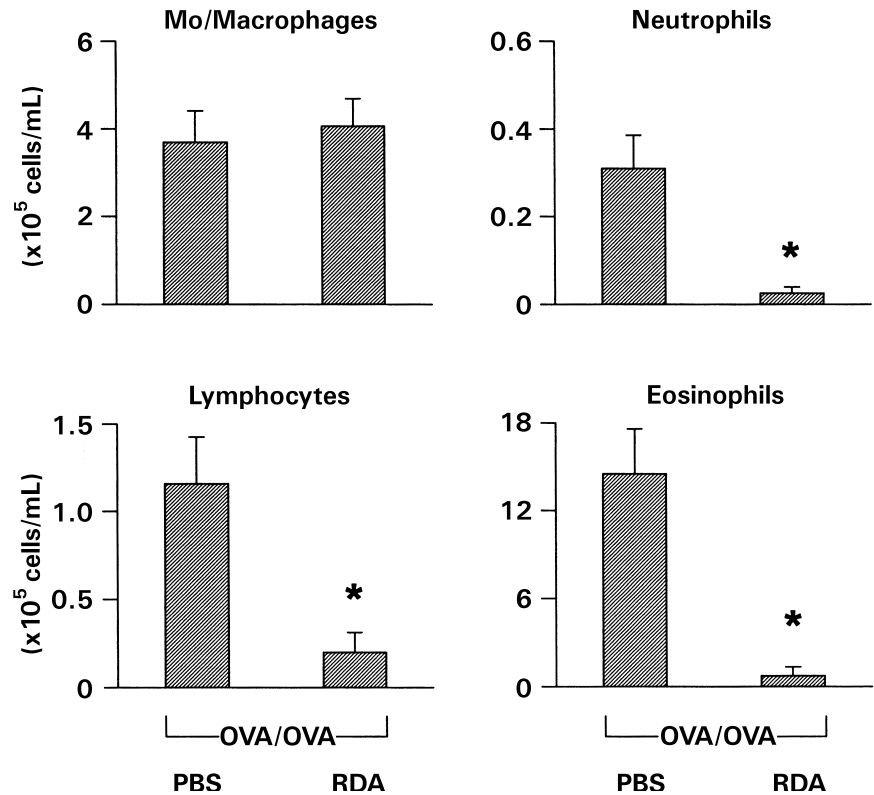


Fig. 6. RDA-mediated inhibition of antigen-induced airways inflammation in IL-12 KO mice. Prior to sensitization, IL-12 KO mice were treated with PBS or RDA (6×10^8 p.f.u.) i.m. Data show changes in inflammatory cells in the BAL 72 h after OVA-aerosol challenge (mean $\pm$ SEM, $n = 5$; unpaired t -test; *, $P < 0.05$).



particularly rhinovirus and RSV, can exacerbate asthma [5,6]. However, the interaction between viral infections, allergic sensitization and asthma in young children remains a controversial subject. This is of great importance as it has been proposed that key events in the aetiology of human allergic disease and the development of asthma, i.e. T-cell selection and the generation of dominant long-term immune responses to aeroallergens, likely occur during the first few years of life [29–31]. In this regard, recent epidemiological data suggest that certain childhood infections may protect against the development of asthmatic and allergic inflammation [7–13].

The present study was designed to investigate whether induction of an immune response to virus concurrent with initial exposure to an allergen (OVA) would affect secondary immune-inflammatory responses in the airways. To this end, the RDA was delivered prior to sensitization with OVA to recreate a situation of initial antigen exposure in the context of an ongoing immune response against virus. Hence, this experimental design investigates the impact of viral infection during sensitization to an allergen, and is not intended to address the effect of viral infections on established airways inflammation. A replication-deficient adenovirus was injected into the hind muscle to achieve a transient and localized viral infection with minimal dissemination to extramuscular sites [27,32]. This study shows that i.m. RDA infection inhibits antigen-induced airways inflammation. As far as the authors are aware, the data presented here provide the first experimental evidence that a viral infection, specifically with adenovirus, at a site distant from the airways, inhibits the development of TH2-driven airways eosinophilic inflammation.

Clearly, IFN γ plays a key role in mediating the inhibitory effect of adenovirus on airways inflammation. Results show that inhibition of airways inflammation by RDA does not occur in IFN γ KO mice, but does in IL-12 KO mice (Figs 5 and 6); IFN γ production independent of IL-12 has previously been observed in other experimental models [33–35]. Flow cytometric analysis of BAL cells after antigen challenge revealed a substantial increase in the proportion of T cells, both CD4⁺ and CD8⁺, expressing IFN γ as detected by intracytoplasmic staining. Additionally, we showed that splenocyte IFN γ production after OVA recall *in vitro* was enhanced, while IL-4 was reduced, in cells obtained from mice treated with RDA, but not with PBS alone prior to sensitization. Taken together, these data support the notion that RDA treatment prior to OVA sensitization may have shifted the OVA response from a TH2- towards a TH1-polarized immune response. That the IFN γ protein content in the BAL was reduced in RDA-treated mice after antigen challenge may appear to contradict the notion that IFN γ plays a critical role in RDA-mediated inhibition of airways inflammation.

However, as shown in Fig. 1, the number of lymphocytes in the BAL of RDA-treated mice was reduced by $\approx 65\%$ compared with PBS-treated mice. Thus, we suggest that the lower level of IFN γ in the BAL of RDA-treated mice reflects the considerably smaller number of lymphocytes in the lungs of these mice.

While this study shows that inhibition of airways eosinophilia by RDA is IFN γ dependent, so far we have been unable to detect increased amounts of IFN γ in the serum after RDA treatment (data not shown). It is, then, plausible that effector cells producing IFN γ in response to RDA locally modify the cytokine environment in which allergic sensitization takes place, thereby skewing the phenotype of the OVA response. This hypothesis is supported by the data, indicating an altered *in vitro* cytokine OVA recall response in the spleen, and an inhibition of the airways inflammatory response after antigen challenge. Furthermore, there is a marked enlargement of the regional lymph nodes (inguinal and lumbar) after RDA treatment, and the authors hypothesize that some of these cells circulate and locally modify allergic sensitization. Currently, experiments to identify the corresponding effector cell type are being intensively pursued in our laboratory.

It is of interest that RDA administration had no effect on the levels of OVA-specific IgE, but remarkably affected airways eosinophilia. B cell-deficient mice — which lack all immunoglobulins including IgE, as well as IgE-deficient mice — have recently been shown to be capable of mounting airways eosinophilic responses of an intensity similar to those in control littermates [36,37]. The findings of the present study further support the notion that the generation of airways eosinophilia is independent of IgE-mediated events.

Given that the effects of RDA are IFN γ -dependent, it is surprising that IgE synthesis was not significantly affected. However, we showed that splenocytes from RDA-treated mice were still capable of producing a substantial amount of IL-4, and this may have been sufficient to induce ϵ isotype switching. It remains to be explored whether repeated viral infections would further shift the response toward TH1 to a point at which IgE synthesis is affected. Experiments to test this hypothesis are underway.

In summary, we have demonstrated that an ongoing RDA-induced immune response at the time of the initial exposure to antigen (OVA) inhibits subsequent antigen-induced airways inflammation, and that this inhibition is IL-12-independent and IFN γ -dependent. These findings support the hypothesis that exposure to allergens in the context of agents that induce a TH1-polarized immune response, such as certain viruses or attenuated intracellular bacteria, may prevent allergic sensitization [7–13]. Understanding the fundamental mechanisms involved in this process may lead to the development of strategies to immunoregulate responses to aeroallergens.

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