

Allergen Immunotherapy Inhibits Airway Eosinophilia and Hyperresponsiveness Associated with Decreased IL-4 Production by Lymphocytes in a Murine Model of Allergic Asthma

Antoon J. M. Van Oosterhout, Betty Van Esch, Gerard Hofman, Claudia L. Hofstra, Ingrid Van Ark, Frans P. Nijkamp, Martien L. Kapsenberg, Huub F. J. Savelkoul, and Frank R. Weller

Department of Pharmacology and Pathophysiology, Utrecht University, Utrecht; Laboratory of Cell Biology and Histology, Academic Medical Center, Amsterdam; Department of Immunology, Erasmus University, Rotterdam; and Department of Pulmonology, Academic Medical Center, Amsterdam, The Netherlands

In the present study, we investigated whether allergen immunotherapy is effective in a murine model with immunologic and pathophysiologic features reminiscent of allergic asthma. Ovalbumin-sensitized mice received increasing (1 μ g to 1 mg) subcutaneous doses of ovalbumin twice a week for 8 wk according to a semirush immunotherapy protocol as used in allergic patients. During immunotherapy, an initial rise in serum levels of ovalbumin-specific antibodies (immunoglobulin [Ig]G₁, IgE, IgG_{2a}) occurred, after which IgE levels decreased sharply concomitant with an increase in IgG_{2a} levels. The increase in IgG_{2a} levels, with the decline in IgE levels, suggests that during immunotherapy interferon- γ production is increased or interleukin (IL)-4 production is decreased. After immunotherapy, inhalation challenge of the mice with ovalbumin revealed almost complete inhibition (98%, $P < 0.01$) of eosinophil infiltration into bronchoalveolar lavage and airway hyperresponsiveness (100% at 320 μ g/kg methacholine, $P < 0.05$) compared with sham-treated animals. In addition, IL-4 production of thoracic lymph node cells stimulated with ovalbumin *in vitro* was largely reduced (60%, $P < 0.05$) after immunotherapy. Thus, effective immunotherapy in this animal model appears to be due to modulation of antigen-specific T cells. Similar effects on airway symptoms and IL-4 production can be obtained within 1 wk by three injections of the highest dose of ovalbumin (1 mg). This animal model will be used as a preclinical model to improve allergen immunotherapy and to gain more insight into the mechanisms involved. **Van Oosterhout, A. J. M., B. Van Esch, G. Hofman, C. L. Hofstra, I. Van Ark, F. P. Nijkamp, M. L. Kapsenberg, H. F. J. Savelkoul, and F. R. Weller. 1998. Allergen immunotherapy inhibits airway eosinophilia and hyperresponsiveness associated with decreased IL-4 production by lymphocytes in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 19:622–628.**

Allergen immunotherapy has been used since 1911 for many allergic disorders (1). Analyses of clinical trials demonstrated a reduction in required medication and of clinical symptoms, such as peak expiratory flow rate and sensitivity to bronchial provocation by allergen (2, 3). In addition, allergen immunotherapy has been demonstrated

to inhibit allergic airway inflammation and airway hyperresponsiveness (2, 3). Recently, Creticos and colleagues (4) demonstrated a reduced seasonal increase in allergen-specific immunoglobulin (Ig)E and reduced skin-test sensitivity to allergens during immunotherapy. However, immunotherapy in asthmatic patients seldom results in complete alleviation of symptoms, and there is always a risk of inducing bronchoconstrictive responses and systemic anaphylaxis (2, 3). Thus, improvement of allergen immunotherapy is needed before widespread application in patients with allergic asthma is considered. The specific immunologic mechanisms by which immunotherapy achieves its effect have not been fully elucidated (5). The induction of "blocking antibodies," downregulation of Th2 lymphocytes and/or upregulation of Th1 lymphocytes, or the induction of CD8⁺ "suppressor" T cells have all been postulated as possible underlying mechanisms (5, 6).

Studies in laboratory animals will be valuable as a pre-

(Received in original form July 29, 1997 and in revised form February 2, 1998)

Address correspondence to: Dr. A. J. M. Van Oosterhout, Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands. E-mail: A.J.M.VanOosterhout@far.ruu.nl

Abbreviations: bovine serum albumin, BSA; bronchoalveolar lavage fluid, BALF; enzyme-linked immunosorbent assay, ELISA; immunoglobulin, Ig; interferon- γ , IFN- γ ; interleukin, IL; lymph nodes, LN; phosphate-buffered saline, PBS.

Am. J. Respir. Cell Mol. Biol. Vol. 19, pp. 622–628, 1998
Internet address: www.atsjournals.org

clinical model to improve allergen immunotherapy and to unravel the mechanisms involved. Recently, we developed a murine model with immunologic and pathophysiologic features reminiscent of allergic asthma. In this model, mice are sensitized with ovalbumin without the use of an adjuvant, leading to ovalbumin-specific IgE antibodies in serum (7). Repeated exposure of sensitized mice to ovalbumin aerosol induced airway inflammation characterized by eosinophil infiltration in lung tissue, trachea, and bronchoalveolar lavage fluid (BALF) and development of airway hyperresponsiveness of methacholine and serotonin (8–10).

In the present study we examined whether a semirush immunotherapy protocol according to human allergen immunotherapy was effective in this animal model. It is demonstrated that ovalbumin immunotherapy induces a shift in ovalbumin-specific antibodies from IgE to IgG_{2a} in serum. Subsequent inhalation challenge with ovalbumin reveals an almost complete inhibition of airway eosinophilia and hyperresponsiveness, which coincides with a reduced production of interleukin (IL)-4 by antigen-specific T cells in the draining lymph nodes. Furthermore, we demonstrate that similar effects can be obtained within 1 wk by three injections of the highest dose of ovalbumin (1 mg).

Materials and Methods

Sensitization and Challenge

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specified pathogen-free male BALB/c mice (6 to 8 wk) were obtained from the Central Animal Laboratory, Utrecht, The Netherlands. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Active sensitization was performed by seven intraperitoneal injections of 10 µg ovalbumin (grade V) in 0.5 ml pyrogen-free saline on alternate days (one injection per day). This sensitization procedure has been shown to induce high titers of total IgE antibodies in serum, of which 80% were ovalbumin specific (7). After sensitization, the animals were divided in two groups of 12 animals. According to semirush allergen immunotherapy protocol as used in allergic patients, one group was treated with increasing (1 µg to 1 mg) subcutaneous doses of ovalbumin twice a week for 8 wk prior to aerosol antigen challenge. The other group was sham treated with saline injections of the same volume (0.2 ml). Two days after the last injection, half the animals from each group were exposed to repeated ovalbumin (2 mg/ml) aerosols once a day for 8 d, and the other half was exposed to saline aerosols. The aerosol was applied in a plexiglas exposure chamber (5 liters) coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment, Richmond, VA; particle size 2 to 3 µm), driven by compressed air at a flow rate of 6 liters/min. Exposure was performed for 5 min and in groups of six animals maximum.

In an additional series of experiments, ovalbumin-sensitized animals were treated by three subcutaneous injections of the highest dose of ovalbumin (1 mg) or saline on alternate days. Two days after the last injection, the animals were challenged with ovalbumin or saline as previously described.

Serum Levels of Ovalbumin-specific Antibodies

A separate group of animals was sensitized as described previously, and blood samples were taken after sensitization and at various days during (sham) immunotherapy. Sera were stored at -20°C until levels of ovalbumin-specific IgE, IgG₁, and IgG_{2a} were determined by enzyme-linked immunosorbent assay (ELISA). To determine serum levels of ovalbumin-specific IgE, 96-well microtiter plates (Falcon 3912 microtest III; Falcon, Lelystad, The Netherlands) were coated overnight at 4°C with 2 µg/ml rat-antimouse IgE (EM 95) (11) diluted in 10 mM phosphate-buffered saline (PBS), pH 7.3. Subsequently, plates were blocked by PBS containing 1% (wt/vol) bovine serum albumin (BSA) and left to incubate for 1 h at room temperature. After multiple washings with PBS containing 0.05% (vol/vol) Tween-20 (PBS-Tween), plates were loaded with a twofold dilution series in RPMI 1640 medium of appropriately prediluted serum samples and twofold dilutions of an ovalbumin-specific reference standard in duplicate. As a standard, pooled serum obtained from multiple ovalbumin-boosted mice was used with an arbitrary concentration of 1,000 U/ml. The plates were incubated for 3 h at room temperature. Ovalbumin-specific IgE was detected with appropriately diluted digoxigenin-coupled ovalbumin prepared with digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid *N*-hydro-succinimide ester according to the manufacturer's instructions (Boehringer Mannheim, Almere, The Netherlands). After washing, plates were incubated for 1 h at room temperature with 150 mU/ml peroxidase-coupled sheep antidigoxigenin Fab fragments (Boehringer Mannheim) diluted in PBS-Tween. Plates were then incubated with 2,2'-azino-bis-(3-ethylbenthiazoline-6-sulfonic acid) in 0.1 M citric acid containing 0.003% peroxide. Plates were read at 419 nm, and IgE concentrations were determined with reference to the standard curve. The detection limit of the ELISA is 0.5 U/ml.

To determine ovalbumin-specific IgG levels, plates were coated with 10 µg/ml ovalbumin in PBS. For detection, 0.3 µg/ml biotinylated rabbit-antimouse IgG₁ (Zymed, San Francisco, CA), or 0.5 µg/ml biotinylated goat-antimouse IgG_{2a} (Southern Biotechnology, Birmingham, AL) and 1.6 µg/ml peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) were used. A reference standard was used with arbitrary units of each isotype of 1,000 U/ml. The detection limit of the ELISA is 0.005 U/ml for IgG₁ and 0.05 U/ml for IgG_{2a}.

Airway Responsiveness *In Vivo*

Airway responsiveness was measured *in vivo* 24 h after the last aerosol exposure using a modified plethysmograph (Buxco, EMKA Technologies, Paris, France) as described by Corry and colleagues (12). In short, mice were anesthetized by intraperitoneal injection of urethane (2 g/kg), and placed on a heated blanket (30°C). The trachea was then cannulated and a small polyethylene catheter was placed in the jugular vein for intravenous administration. Spontaneous breathing of the animals was suppressed by intravenous injection of tubocurarine chloride (3.3 mg/kg). When the breathing stopped, the tracheal cannula was attached to a ventilator (C. F. Palmer, London, UK). The inflation

volume of the ventilator was 0.8 ml, of which the mice inhaled approximately 0.15 ml per breath with a rate of 200 breaths per minute. Under these conditions, mice maintain physiologic arterial blood gas parameters (data not shown). Changes in resistance were measured by use of a plethysmograph coupled to a pressure transducer (M45; Validyne Engineering Corp., Northridge, CA). By use of a pulmonary mechanics analyzer (Buxco), lung resistance (R_L) was measured by quantitating change in tracheal pressure (ΔP_T) divided by change in flow ($\Delta \dot{V}$) at points of equal volume (70% tidal volume). Changes in tracheal pressure were measured using a pressure transducer connected to the tracheal ventilation cannula; changes in flow were measured by use of a pressure transducer connected to the plethysmograph (pressure changes were calibrated to changes in volume over the physiologic range studied). At time intervals of at least 4 min and after the response had returned to baseline level, doses of methacholine ranging from 40 to 1,280 $\mu\text{g}/\text{kg}$ were administered via the jugular catheter. Concentrations of methacholine were prepared in saline and kept on ice for the duration of the experiment. For each dose of methacholine, the increase in airway resistance was measured at its peak and expressed in $\text{cm H}_2\text{O}/\text{ml}/\text{s}$.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed in the same animals that were used for airway hyperresponsiveness measurements. In pilot experiments, it was observed that combining the two techniques had no effect on total number of cells derived from lavage or on the appearance of different cell types. After completion of the dose-response curve to methacholine, the animals were lavaged five times through the tracheal cannula with 1-ml aliquots of pyrogen-free saline warmed to 37°C. The BALF was kept on ice until further processing. The BALF cells were washed with PBS (400 \times g, 4°C, 5 min) and the pellet was resuspended in 150 μl PBS. Total numbers of BALF cells were counted in a Burkert-Türk chamber (Omnilabo, Breda, The Netherlands). For differential BALF cell counts, cytopspin preparations were made and stained with Diff-Quik (Merz & Dade A.G., Dudingon, Switzerland). After they were coded, all cytopspin preparations were evaluated by one observer using oil immersion microscopy (magnification: $\times 1,000$). Cells were identified and differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology. Per cytopspin preparation, at least 200 cells were counted and the absolute number of each cell type was calculated.

Effect of Immunotherapy on Cytokine Production

At the end of the experiment, thoracic lymph nodes (LN) draining the lungs were removed from the paratracheal and parabronchial region, transferred to cold PBS, and gently homogenized on a 70- μm cell strainer (Falcon) to obtain a single-cell suspension. The cells were washed and resuspended in culture medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 1% glutamax I, 50 $\mu\text{g}/\text{ml}$ gentamycin, and 50 μM β -mercapto-ethanol). Cells were cultured in round-bottom 96-well plates (Costar, Badhoevedorp, The Netherlands) at a concentration of 2×10^5

cells per well in a volume of 200 μl . In preliminary experiments, the optimal conditions for ovalbumin-induced cytokine production by thoracic LN cells were established (C. L. Hofstra, unpublished observations). The cells were cultured for 5 d with plate-bound anti-CD3 (clone 17A2, 50 $\mu\text{g}/\text{ml}$, coating for 18 h at 4°C) (13), ovalbumin (10 $\mu\text{g}/\text{well}$), or medium only. The cells were cultured at 37°C with 5% CO_2 in humidified air. Each *in vitro* stimulation was performed in triplicate. Supernatants were harvested, pooled per mouse, and kept at -20°C until IL-4 and interferon- γ (IFN- γ) levels were determined by ELISA.

IL-4 and IFN- γ ELISA

Flat-bottom microplates (96 wells, Maxisorp; Nunc, Life Technologies, Breda, The Netherlands) were coated with coat antibody (1 $\mu\text{g}/\text{ml}$) in PBS (anti-IL-4, 11B11; Pharmingen, San Diego, CA) or PBS with 38 mM Na_2CO_3 and 43 mM KH_2PO_4 (anti-IFN- γ , R4-6A2; Pharmingen) at 4°C for 18 h. After coating, plates were washed with PBS containing 0.05% Tween-20 and blocked with ELISA buffer (2 mM ethylenediamine tetraacetic acid; 136.9 mM NaCl; 50 mM Tris; 0.5% BSA; and 0.05% Tween-20, pH 7.2) at room temperature for 1 h while being shaken gently. After washing, supernatant samples (diluted 1:1 with ELISA buffer) and recombinant mouse (rm)IL-4 (Genentech Inc., South San Francisco, CA) or rmIFN- γ (Genentech) were applied and incubation was continued at room temperature for 2 h. Thereafter, plates were washed and the second biotinylated antibody diluted in ELISA buffer was added followed by incubation at room temperature for 1.5 h while being shaken. The second antibodies (1 $\mu\text{g}/\text{ml}$) applied were antimouse IL-4 monoclonal antibodies (BVD6-24G2; Pharmingen) and antimouse IFN- γ (XMG1.2; Pharmingen). After washing, streptavidin-peroxidase (0.3 $\mu\text{g}/\text{ml}$; Jackson, West Grove, PA) was added, and incubation was performed at room temperature for 1 h. After the plates were washed, 0.4 mg/ml o-phenylenediamine-dihydrochloride in PBS containing 0.012% hydrogen peroxide was added. After approximately 30 min the reaction was stopped by adding 4 M H_2SO_4 . Subsequently, optical density was measured at 492 nm. The detection limits of the ELISAs were 16 pg/ml for IL-4 and 160 pg/ml for IFN- γ .

Chemicals

Ovalbumin, 2,2'-azino-bis-(3-ethylbenthiazoline-6-sulfonic acid), and o-phenylenediamine were purchased from the Sigma Chemical Company (St. Louis, MO), urethane and methacholine from Janssen Chimica (Beerse, Belgium), and tubocurarine chloride from Noguepha (Alkmaar, The Netherlands).

Data Analysis

Data are expressed as means \pm SEM, and comparisons between groups were made using Student's *t* test. A difference was considered to be significant when $P < 0.05$.

Results

Serum Antibody Levels

Ovalbumin sensitization increased serum levels of ovalbumin-specific IgG₁ and IgE, but not IgG_{2a}, which reached a

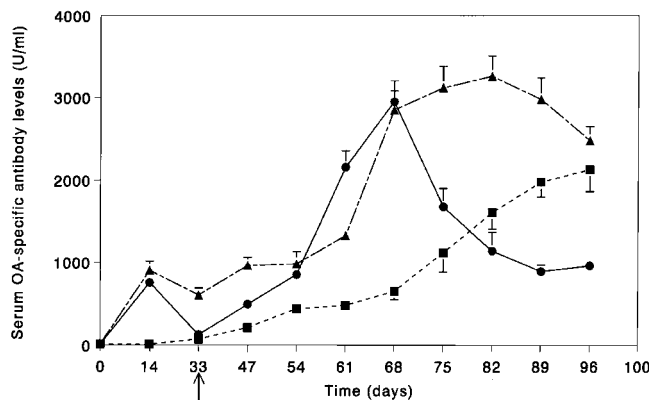


Figure 1. Serum antibody levels during ovalbumin immunotherapy. Serum levels of ovalbumin-specific IgE (circles), IgG₁ (triangles), and IgG_{2a} (squares) in arbitrary units (U/ml) at various days after sensitization and during ovalbumin immunotherapy. Arrow indicates the start of immunotherapy. Results are expressed as means ± SEM of six animals.

maximum level after 14 d (Figure 1). During immunotherapy, serum levels of ovalbumin-specific IgE, IgG₁, and IgG_{2a} increased until Day 68. Thereafter, serum IgE levels showed a sharp drop, whereas IgG_{2a} levels continued to increase. Serum levels of ovalbumin-specific IgG₁ remained elevated after Day 68 of immunotherapy. These changes in antibody levels were reflected in the levels of total antibody of each isotype (results not shown). During sham immunotherapy, ovalbumin-specific IgG₁ levels rose to 920 ± 180 U/ml on Day 54 and tapered off thereafter, whereas ovalbumin-specific IgG_{2a} levels remained undetectable. Ovalbumin-specific IgE levels slowly rose from Day 33 onward to 430 ± 270 U/ml.

In a subsequent experiment, animals were treated with three injections of the highest dose of ovalbumin (1 mg) and repeatedly challenged with either saline or ovalbumin. In sham-treated mice, ovalbumin challenge induced a significant increase in the serum levels of ovalbumin-specific IgE and IgG₁, but not IgG_{2a}, compared with saline-challenged animals (Table 1). In saline-challenged control animals, high-dose immunotherapy strongly increased the serum levels of IgG₁ and IgG_{2a} more than 25-fold and

10-fold, respectively, whereas serum levels of IgE only increased twofold (Table 1). After high-dose immunotherapy, ovalbumin challenge induced a significant decrease in serum levels of ovalbumin-specific IgE and IgG₁ and a significant increase in IgG_{2a} compared with saline-challenged animals (Table 1).

Bronchoalvolar Cell Infiltration

In sham-treated mice, ovalbumin challenge induced a substantial infiltration of eosinophils into the BALF compared with saline-challenged animals (ovalbumin, 8.6 ± 1.4 × 10⁵ cells versus saline, 0.02 ± 0.01 × 10⁵ cells) (Figure 2A). After immunotherapy, eosinophil infiltration was largely reduced (98%, P < 0.01), although ovalbumin challenge still induced a small increase (P < 0.01) in eosinophil number compared with saline-challenged control animals (ovalbumin, 0.15 ± 0.03 × 10⁵ cells versus saline, 0.04 ± 0.01) (Figure 2A).

No significant differences in eosinophils were observed between saline-challenged mice in the immunotherapy group compared with the sham-treated group. Furthermore, no significant differences were observed between total numbers of leukocytes and mononuclear cells in BALF between the four different groups (Figure 2A). No neutrophils were observed in the BALF of either group of animals. Similar inhibitory effects (90%, P < 0.01) on BALF eosinophil numbers were observed after high-dose immunotherapy (Figure 2B).

Airway Responsiveness to Methacholine

In sham-treated mice, ovalbumin challenge significantly potentiated the increase in airway resistance after intravenous administration of methacholine from 320 to 1,280 µg/kg, compared with saline-challenged mice (results not shown). The potentiation amounted to 510% at 320 µg/kg (P < 0.05), 220% at 640 µg/kg (P < 0.01), and 165% at 1,280 µg/kg (P < 0.05). In Figure 3A, the responses of the groups to 320 µg/kg methacholine are presented. Immunotherapy completely inhibited the ovalbumin-induced airway hyperresponsiveness (Figure 3A) back to the response in sham-treated, saline-challenged mice.

In an additional experiment, sensitized mice were treated with three injections of the highest dose of ovalbumin and challenged afterward. This high-dose immuno-

TABLE 1
Serum ovalbumin-specific antibody levels of sensitized mice after immunotherapy with three injections of ovalbumin (1 mg) or sham treatment (saline) and subsequent repeated challenge with either ovalbumin or saline

| Ig Isotype (U/ml) | Treatment Groups | | | |
|-------------------|------------------|-----------------|-------------------------------|--------------------------|
| | Sham | | Immunotherapy (3 × high dose) | |
| | Saline | Ovalbumin | Saline | Ovalbumin |
| IgE | 3,735 ± 656 | 11,449 ± 1,129* | 6,828 ± 146 [†] | 5,080 ± 213* |
| IgG ₁ | 589 ± 33 | 1,452 ± 167* | 14,764 ± 542 [†] | 11,946 ± 394* |
| IgG _{2a} | 94 ± 22 | 137 ± 14 | 1,102 ± 119 [†] | 1,511 ± 111 [†] |

Results are expressed as the mean ± SEM of 7 to 12 animals.

*P < 0.01 and [†]P < 0.05 compared with saline-challenged mice of the same group and determined by Student's *t* test.

[‡]P < 0.01 compared with sham-treated, saline-challenged mice.

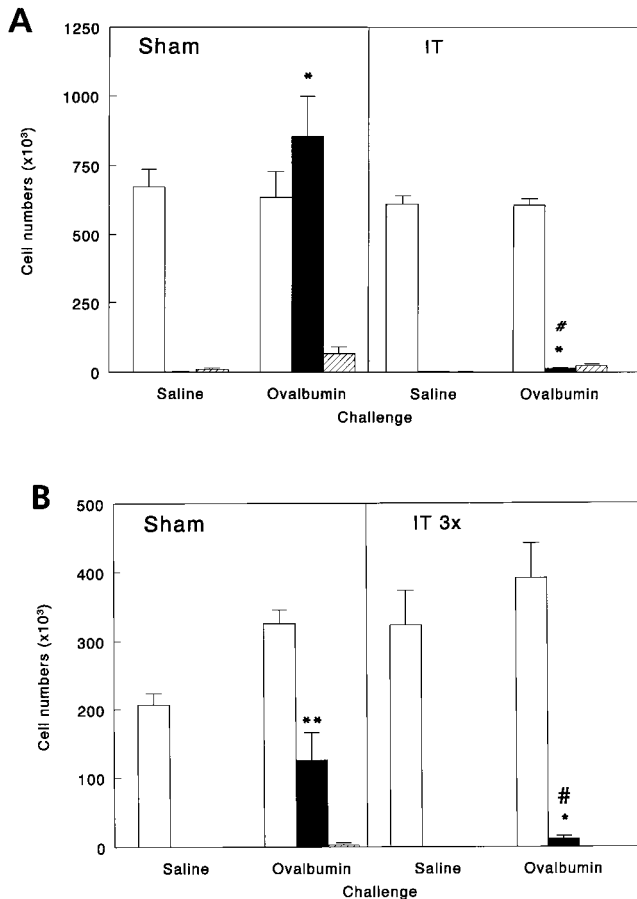


Figure 2. Ovalbumin-induced leukocyte infiltration into BALF. Numbers of macrophages (open bars), eosinophils (closed bars), and lymphocytes (hatched bars) in BALF of ovalbumin-sensitized, sham-treated animals (left, sham) or after immunotherapy (right, IT) using a semirush protocol (A) or high-dose immunotherapy (B) and repeatedly challenging with saline or ovalbumin. Results are expressed as means \pm SEM of six animals. * $P < 0.01$ compared with saline-challenged mice of the same group and determined by Student's *t* test. # $P < 0.01$ compared with sham-treated, ovalbumin-challenged mice and determined by Student's *t* test.

therapy completely inhibited the ovalbumin-induced airway hyperresponsiveness to methacholine when compared with sham-treated animals (Figure 3B).

Effect of Immunotherapy on Cytokine Synthesis

Cultures of thoracic LN cells isolated from sham-treated, ovalbumin-challenged mice produced significantly ($P < 0.01$) increased levels of IL-4 compared with saline-challenged mice (ovalbumin, 802 ± 165 pg/ml versus saline, 107 ± 36 pg/ml) after antigen-specific stimulation *in vitro* (Figure 4A). Immunotherapy significantly ($P < 0.05$) decreased the IL-4 production of LN cells with approximately 60% in ovalbumin-challenged animals. However, after immunotherapy, cultures of LN cells isolated from ovalbumin-challenged mice continued to produce significantly ($P < 0.05$) increased levels of IL-4 compared with saline-challenged mice (ovalbumin, 324 ± 105 pg/ml ver-

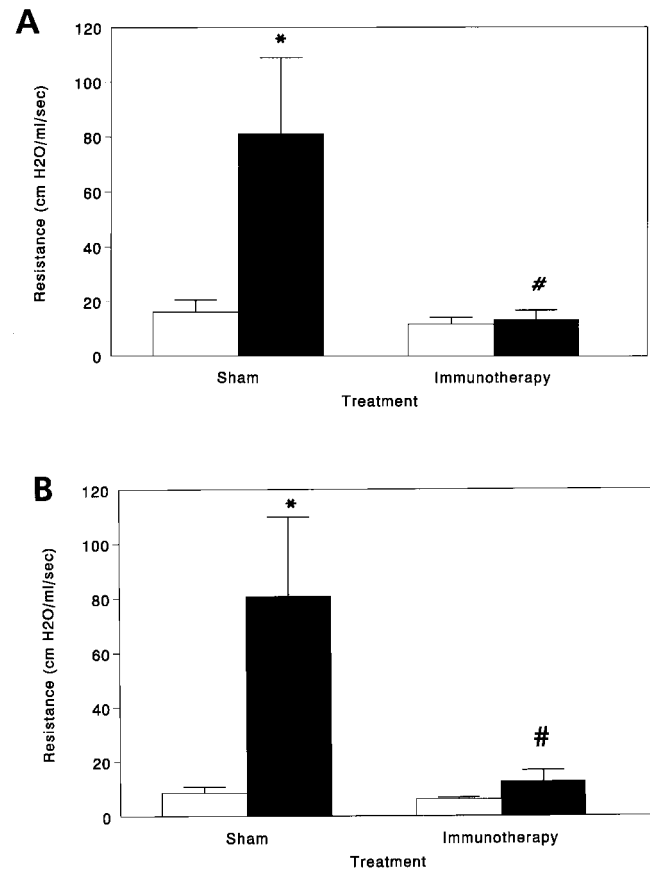


Figure 3. Ovalbumin-induced airway hyperresponsiveness. Increase in airway resistance after intravenous injection of $320 \mu\text{g/kg}$ methacholine in ovalbumin-sensitized animals after either sham treatment (sham) or immunotherapy using a semirush protocol (A) or high-dose immunotherapy (B) and repeatedly challenging with saline (open bars) or ovalbumin (black bars). Results are expressed as means \pm SEM of six animals. * $P < 0.05$ compared with saline-challenged mice of the same group and determined by Student's *t* test. # $P < 0.05$ compared with sham-treated, ovalbumin-challenged mice and determined by Student's *t* test.

sus saline, 68 ± 19 pg/ml) after antigen-specific stimulation *in vitro* (Figure 4A). No difference was observed in IL-4 production by LN cells between the sham treatment and immunotherapy in saline-challenged animals.

In an additional experiment, sensitized mice were treated with three injections of the highest dose of ovalbumin and subsequently challenged. After high-dose immunotherapy, LN cultures from ovalbumin-challenged animals produced significantly less IL-4 (85%, $P < 0.05$) upon ovalbumin restimulation compared with sham-treated mice (Figure 4B).

Ovalbumin stimulation did not induce detectable levels of IFN- γ in the LN cultures of the different groups. Polyclonal stimulation of LN cells with anti-CD3 induced detectable levels of IFN- γ in the supernatants. However, no significant differences in the levels of IFN- γ or IL-4 were observed between different groups after anti-CD3 stimulation (results not shown).

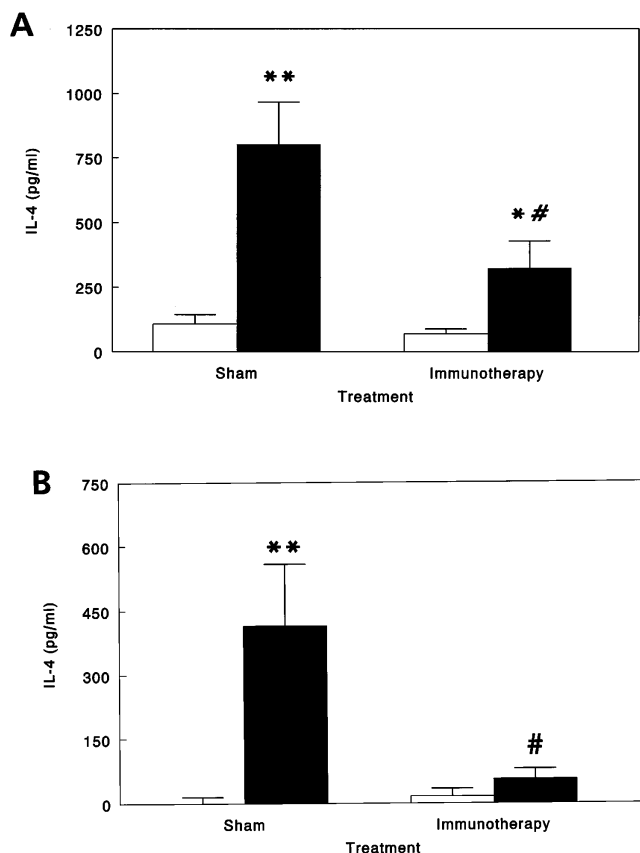


Figure 4. IL-4 production by thoracic lymph node cells. Levels of IL-4 in supernatants of thoracic lymph node cells isolated from ovalbumin-sensitized mice after either sham treatment (*sham*) or immunotherapy using a semirush protocol (*A*) or high-dose immunotherapy (*B*) and repeatedly challenging with saline (*white bars*) or ovalbumin (*black bars*). Results are expressed as means \pm SEM of six animals. * $P < 0.05$ and ** $P < 0.01$ compared with saline-challenged mice of the same group and determined by Student's *t* test. # $P < 0.05$ compared with sham-treated, ovalbumin-challenged mice and determined by Student's *t* test.

Discussion

Here, we demonstrated that immunotherapy according to an established human semirush allergen immunotherapy protocol induces a shift in serum antibody isotypes and is effective in reducing airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. Interestingly, similar effects on airway symptoms and IL-4 production can be obtained within 1 wk by three injections of the highest dose of ovalbumin (1 mg). The mechanism by which immunotherapy achieves these beneficial effects remains speculative.

During the first month of immunotherapy, we observed a rise in serum levels of ovalbumin-specific antibodies. Thereafter, IgE levels dropped sharply, whereas IgG_{2a} levels continued to increase. Our data on a shift in serum antibody isotypes are similar to the observations in humans during allergen immunotherapy (14, 15). It has been suggested that the increased levels of IgG antibodies compete with IgE for allergen and thus may block IgE-mediated events (15).

The shift in antibody isotypes is probably a reflection of an altered T-lymphocyte response. IFN- γ is an important switch factor for IgG_{2a} synthesis by murine B lymphocytes, and low concentrations of IFN- γ inhibit IL-4-induced increases in IgE synthesis (16). In addition, IL-4 is much less important for the production of IgG₁ (16). Thus, the concomitant increase in IgG_{2a} levels in conjunction with the decline in IgE levels suggests that during immunotherapy IFN- γ production is increased or IL-4 production is decreased. One possibility could be that immunotherapy induced a shift in the cytokine profile of antigen-specific lymphocytes from a Th2- to a Th1-type response. Indeed, we observed a diminished ovalbumin-induced IL-4 production by lymphocytes obtained from the draining lymph nodes after ovalbumin challenge. This reduction of IL-4 production is antigen-specific because polyclonal stimulation induced similar levels of IL-4. However, after immunotherapy, IFN- γ levels were not increased in lymphocyte cultures stimulated with antigen *in vitro*. These data suggest that immunotherapy affects the cytokine profile of antigen-specific Th2-lymphocytes but does not induce a Th1 shift. Interestingly, in human atopic patients, allergen immunotherapy has been shown to decrease allergen-induced IL-4 production by CD4⁺ T lymphocytes (6). However, McHugh and colleagues (17) reported increases in IFN- γ and decreases in IL-4 production in short-term peripheral blood mononuclear cell cultures following immunotherapy.

In humans, it has been demonstrated that during allergen immunotherapy CD8⁺ suppressor T cells are generated (18). These CD8⁺ cells may be a source of IFN- γ , leading to the rise in IgG_{2a} levels. Although we did not observe an increased IFN- γ production in the draining LN after immunotherapy, it remains possible that IFN- γ -producing CD4⁺ or CD8⁺ lymphocytes reside at another location in the body. More studies are necessary to examine this possibility in the mouse model.

After immunotherapy, antigen-induced infiltration of eosinophils into BALF and airway hyperresponsiveness were almost completely inhibited. The mechanism by which immunotherapy affects these effector responses could be similar to those responsible for the antibody isotype shift, that is, a shift in the IL-4/IFN- γ ratio produced by antigen-specific T lymphocytes. Interestingly, the effects of immunotherapy appear to be antigen-specific because immunotherapy with an unrelated antigen keyhole-limpet hemocyanin did not affect the antigen-induced eosinophil infiltration (A. J. M. Van Oosterhout, unpublished observation). In humans it has been shown that immunotherapy results in a decrease in CD4⁺ cell recruitment and a reduction in local eosinophilia during allergen-induced late responses in the nose (19). These changes were accompanied by increases in allergen-induced IFN- γ mRNA-positive cells, whereas IL-4⁺ and IL-5⁺ cells remained unchanged. Interestingly, CD8⁺ lymphocytes producing IFN- γ have been demonstrated to be negative regulatory cells of airway hyperresponsiveness in a comparable murine model (20). In addition, we (21) and others (22, 23) have demonstrated that IFN- γ administration during antigen challenge can also prevent both airway eosinophilia and hyperresponsiveness.

In humans, different immunotherapy protocols exist in

which the main difference is the time before high doses of allergen are administered (24). Immediate treatment with high doses would result in fatal anaphylactic reactions. However, the therapeutic effects of immunotherapy are probably due to the high-dose treatment. In the present study, we examined the effects of treatment with three high doses of ovalbumin on antigen-induced symptoms. Unlike humans, mice do not show severe anaphylactic reactions upon injection of high doses of ovalbumin when the doses are given subcutaneously. High-dose immunotherapy induced a large increase in serum levels of ovalbumin-specific IgG₁ and IgG_{2a} and prevented the increase in IgE during antigen challenge. Furthermore, antigen-induced infiltration of eosinophils into BALF and airway hyperresponsiveness were largely inhibited, concomitant with decreased IL-4 production of lymphocytes upon ovalbumin restimulation. These data closely resemble the effects of the semirush immunotherapy protocol. It is tempting to speculate that very short-term and high-dose immunotherapy might be of therapeutic relevance in humans in anaphylactic reactions could be prevented by methods such as anti-IgE treatment (25) or the use of engineered recombinant allergens lacking reactivity to IgE (26).

It can be concluded that immunotherapy in a murine model of allergic asthma is effective in reducing airway eosinophilia and hyperresponsiveness, which is associated with a decreased IL-4 production by lymphocytes and a shift in serum antibody isotypes. Although we treated the mice prior to the induction of airway symptoms and not in ongoing airway disease, this model closely resembles the effects observed during allergen immunotherapy in humans. Thus, this animal model may be valuable as a pre-clinical model to improve allergen immunotherapy and to unravel the precise mechanisms involved in the beneficial effects.

Acknowledgments: Financial support was obtained for B. Van Esch (AF 95.58), C. L. Hofstra (AF 93.63), and I. Van Ark (AF 93.63) from the Netherlands Asthma Foundation.

References

- Noon, L., and B. C. Cantab. 1911. Prophylactic inoculation against hay fever. *Lancet* 1:1572-1574.
- Abramson, M. J., R. M. Puy, and J. M. Weiner. 1995. Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am. J. Respir. Crit. Care Med.* 151:969-974.
- Bousquet, J., and F. B. Michel. 1994. Specific immunotherapy in asthma: is it effective? *J. Allergy Clin. Immunol.* 94:1-11.
- Creticos, P. S., C. E. Reed, P. S. Norman, J. Khoury, N. F. Adkinson, Jr., C. R. Buncher, W. W. Busse, R. K. Bush, J. Gadde, J. T. Li, H. B. Richerson, R. R. Rosenthal, W. R. Solomon, P. Steinberg, and J. W. Yunginger. 1996. Ragweed immunotherapy in adult asthma. *N. Engl. J. Med.* 334:501-506.
- McHugh, S. 1996. Immunological responses to immunotherapy. *Clin. Exp. Allergy* 26:1101-1104.
- Secrist, H., C. J. Chelen, Y. Wen, J. D. Marshall, and D. T. Umetsu. 1993. Allergen immunotherapy decreases interleukin-4 production in CD4⁺ T-cells from allergic individuals. *J. Exp. Med.* 178:2123-2130.
- Hessel, E. M., A. J. M. Van Oosterhout, C. L. Hofstra, J. J. De Bie, J. Garsen, H. Van Loveren, A. K. C. P. Verheyen, H. F. J. Savelkoul, and F. P. Nijkamp. 1995. Bronchoconstriction and airway hyperresponsiveness after ovalbumin inhalation in sensitized mice. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* 293:401-412.
- Hessel, E. M., A. J. M. Van Oosterhout, C. L. Hofstra, J. Garsen, H. Van Loveren, H. F. J. Savelkoul, and F. P. Nijkamp. 1994. Repeated ovalbumin inhalation causes bronchial hyperresponsiveness and eosinophil infiltration in sensitized mice. *Am. Rev. Respir. Dis.* 149:A754. (Abstr.)
- Hessel, E. M., A. J. M. Van Oosterhout, I. Van Ark, B. Van Esch, G. Hofman, H. Van Loveren, H. F. J. Savelkoul, and F. P. Nijkamp. 1997. Development of airway hyperresponsiveness is dependent of IFN- γ and independent of eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* 16:325-335.
- De Bie, J. J., E. M. Hessel, I. Van Ark, B. Van Esch, G. Hofman, F. P. Nijkamp, and A. J. M. Van Oosterhout. 1996. Effect of dexamethasone and endogenous corticosterone on airway hyperresponsiveness and eosinophilia in the mouse. *Br. J. Pharmacol.* 119:1484-1490.
- Van Halteren, A. G. S., M. J. F. Van Der Cammen, J. Biewenga, H. F. J. Savelkoul, and G. Kraal. 1997. IgE and mast cell responses on intestinal allergen exposure: a murine model to study the onset of food allergy. *J. Allergy Clin. Immunol.* 99:94-99.
- Corry, D. B., H. G. Folkesson, M. L. Warnock, D. J. Erle, M. A. Matthay, J. P. Wiener-Kronish, and R. M. Locksley. 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperactivity. *J. Exp. Med.* 183:109-117.
- Vossen, A. C., G. J. Tibbe, M. J. Kroos, J. G. Van De Winkel, R. Benner, and H. F. Savelkoul. 1995. Fc receptor binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects. *Eur. J. Immunol.* 25:1492-1496.
- Lichtenstein, L. M., K. Ishizaka, P. S. Norman, A. K. Sobotka, and B. M. Hill. 1973. IgE antibody measurements in ragweed hayfever. Relationship to clinical severity and the results of immunotherapy. *J. Clin. Invest.* 52:472-482.
- Golden, D. B. K., D. A. Meyers, A. Kagey-Sobotka, M. D. Valentine, and L. M. Lichtenstein. 1982. Clinical relevance of venom-specific immunoglobulin G antibody level during immunotherapy. *J. Allergy Clin. Immunol.* 69:489-493.
- Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145-173.
- McHugh, S. M., J. Deighton, A. G. Stewart, P. J. Lachman, and P. W. Ewan. 1995. Bee venom immunotherapy induces a shift in cytokine responses from a Th2 to a Th1 dominant pattern: comparison of rush and conventional therapy. *Clin. Exp. Allergy* 25:828-838.
- Rocklin, R. E., A. L. Sheffer, D. R. Greineder, and K. L. Melmon. 1980. Generation of antigen-specific suppressor cells during allergy desensitization. *N. Engl. J. Med.* 302:1213-1219.
- Durham, S. R., S. Ying, V. A. Varney, M. R. Jacobson, R. M. Sudderick, I. S. Mackay, A. B. Kay, and Q. A. Hamid. 1996. Grass pollen immunotherapy inhibits allergen-induced infiltration of cells expressing messenger RNA for interferon- γ . *J. Allergy Clin. Immunol.* 97:1356-1365.
- Renz, H., G. Lack, J. Saloga, R. Schwinger, K. Bradley, J. Loader, A. Kupfer, G. L. Larsen, and E. W. Gelfand. 1994. Inhibition of IgE production and normalization of airways responsiveness by sensitized CD8 T cells in a mouse model of allergen-induced sensitization. *J. Immunol.* 152:351-360.
- Hofstra, C. L., I. Van Ark, G. Hofman, P. M. Jardieu, F. P. Nijkamp, and A. J. M. Van Oosterhout. 1996. Differential effects of endogenous and exogenous IFN- γ on IgE, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. *Am. J. Respir. Crit. Care Med.* (In press)
- Lack, G., K. L. Bradley, E. Hamelman, H. Renz, J. Loader, D. Y. M. Leung, G. Larsen, and E. W. Gelfand. 1996. Nebulized IFN- γ inhibits the development of secondary allergic responses in mice. *J. Immunol.* 157:1432-1439.
- Iwamoto, I., H. Nakajima, H. Endo, and S. Yoshida. 1993. Interferon- γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J. Exp. Med.* 177:573-576.
- Nicklas, R. A., I. L. Bernstein, J. Blessing-Moore, S. M. Fineman, A. A. Gutman, R. E. Lee, J. T. Li, W. E. Berger, and S. L. Spector. 1996. Practice parameters for allergen immunotherapy. *J. Allergy Clin. Immunol.* 6:1001-1011.
- Boulet, L. P., K. R. Chapman, J. Cote, S. Kalra, R. Bhagat, V. A. Swystun, M. Laviolette, L. D. Cleland, F. Deschesnes, J. Q. Su, A. Devault, R. B. Fick, and D. W. Cockcroft. 1997. Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *Am. J. Respir. Crit. Care Med.* 155:1835-1840.
- Takai, T., T. Yokota, M. Yasue, C. Nishiyama, T. Yuuki, A. Mori, H. Okudaira, and Y. Okumura. 1997. Engineering of the major house dust mite allergen Der f2 for allergen-specific immunotherapy. *Nature Biotechnol.* 15:754-758.